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(54) Title: **PHARMACOLOGICAL VITREOLYSIS**



(57) Abstract: A method of treating or preventing a disorder, or a complication of a disorder, of an eye of a subject comprising contacting a vitreous and/or aqueous humor with a composition comprising a truncated form of plasmin comprising a catalytic domain of plasmin (TPCD). TPCDs include, but are not limited to, miniplasmin, microplasmin and derivatives and variants thereof. The methods of the invention can be used to reduce the viscosity of the vitreous, liquefy the vitreous, induce posterior vitreous detachment, reduce hemorrhagic blood from the eye, clear or reduce materials toxic to the eye, clear or reduce intraocular foreign substances from the eye, increase diffusion of a composition administered to an eye, reduce extraretinal neovascularization and any combinations thereof. The method can be used in the absence of, or as an adjunct to, vitrectomy.

## **PHARMACOLOGICAL VITREOLYSIS**

### **FIELD OF THE INVENTION**

The present invention relates generally to methods of treating or preventing a disorder, or a complication of a disorder, of the mammalian eye. More specifically,  
5 the present invention relates to the use of a truncated plasmin protein comprising a catalytic domain in methods of treating or preventing a disorder, or a complication of a disorder, of the mammalian eye.

### **BACKGROUND OF THE INVENTION**

The adult human eye is a slightly asymmetrical sphere with an approximate  
10 sagittal diameter of 24 to 25 mm, a transverse diameter of 24 mm, and a volume of about 6.5 cc. The human eye can be divided into three different layers namely, an external layer, an intermediate layer and an internal layer. The external layer of the eye consists of the sclera, which is often referred to as the "white of the eye," and the cornea, which covers the front of the eye. The intermediate layer is divided into an  
15 anterior portion and a posterior portion; the anterior portion consists of the circular pigmented iris, the crystalline lens and ciliary body, while the posterior portion consists of the choroid layer. The internal layer consists of the retina, which is the sensory part of the eye. The retina is essentially a layer of nervous tissue, which runs along the inside rear surface of the choroid layer and can be divided into an optic  
20 portion and a non-optic portion. The optic portion, which participates in the visual mechanism, contains the rods and cones that are the effectual organs of vision.

The human eye can also be divided into three chambers. The anterior chamber between the cornea and the iris, and the posterior chamber between the iris and the crystalline lens, are filled with aqueous humor. In contrast, the vitreous chamber  
25 between the crystalline lens and the retina is filled with a more viscous liquid, called the vitreous (also known as the vitreous body or vitreous humor). The vitreous humor in a normal eye is a clear gel occupying about 80% of the volume of the eyeball. Light that enters the eye through the cornea, pupil, and lens, is transmitted through the vitreous to the retina.

The vitreous humor of a normal human eye is a gel that is roughly 99% water and 1% macromolecules. These macromolecules include a network of collagen fibrils, hyaluronic acid, soluble glycoproteins, sugars and other low molecular weight metabolites. Type II collagen is the principal fibrillar collagen of the vitreous, but the vitreous also contains collagen types V, IX, and XI. The posterior portion of the vitreous body, the posterior hyaloid surface (also known as the posterior vitreous cortex), is in direct contact with the inner retinal surface most prominently at the vitreous base, optic disc, and along the major retinal vessels. Normal adhesion of the vitreous to the retina is mediated by cellular and molecular interactions between the posterior vitreous cortex and the inner limiting membrane (ILM) of the retina. The ILM is essentially the basement membrane of retinal Mueller cells. The ILM contains collagen types I and IV, glycoproteins such as laminin and fibronectin and other glycoconjugates. These components are thought to bridge and bind collagen fibers between the vitreous and the ILM.

With age, the vitreous humor changes from gel to liquid and as it does so it gradually shrinks and separates from the ILM of the retina. This process is known as "posterior vitreous detachment" (PVD) and is a normal occurrence after age 40. However, degenerative changes in the vitreous may also be induced by pathological conditions such as diabetes, Eale's disease and uveitis. Also, PVD may occur earlier than normal in nearsighted people and in those who have had cataract surgery. Usually, the vitreous makes a clean break from the retina. Occasionally, however, the vitreous adheres tightly to the retina in certain places. These small foci of resisting, abnormally firm attachments of the vitreous can transmit great tractional forces from the vitreous to the retina at the attachment site. This persistent tugging by the vitreous often results in horseshoe-shaped tears in the retina. Unless the retinal tears are repaired, vitreous fluid can seep through this tear into or underneath the retina and cause a retinal detachment, a very serious, sight-threatening condition. In addition, persistent attachment between the vitreous and the ILM can result in bleeding from rupture of blood vessels, which results in the clouding and opacification of the vitreous.

The development of an incomplete PVD has an impact on many vitreoretinal diseases including vitreomacular traction syndrome, vitreous hemorrhage, macular holes, macular edema, diabetic retinopathy, diabetic maculopathy and retinal

detachment. Thus, an important goal of vitreous surgery is to separate the vitreous from the retina in a manner that prevents vitreous traction.

In order to remove the vitreous from the eye, a microsurgical procedure called vitrectomy is usually performed. In this procedure the vitreous is removed from the eye with a miniature handheld cutting device while simultaneously replacing the removed vitreous with saline solution to prevent collapse of the eye. Surgical removal of the vitreous using this method is highly skill-dependent, and complete removal of the cortical vitreous remains a difficult task. Furthermore, mechanical vitrectomy carries the risk of complications such as scarring, tearing and other damage to the retina. Obviously, such damage is highly undesirable as it can compromise the patient's vision after surgery.

Thus, alternative methods to remove the vitreous from the retina have been the focus of recent investigation. Such methods have explored the use of enzymes and chemical substances, which can be used to induce/promote liquefaction of the vitreous and/or separation of the vitreoretinal interface (PVD). These approaches, which are referred to as "pharmacological vitrectomy," have included several proteolytic enzymes such as alpha-chymotrypsin, hyaluronidase, bacterial collagenase, chondroitinase and dispase, which have been injected intravitreally in experimental and/or clinical trials to induce PVD. However, most of these techniques do not release the posterior hyaloid from the ILM completely or without complications. In addition, in several of these cases, the risk of adverse reactions is high. For example, the use of bacterial proteases in mammalian systems generates an immune response, which leads to proliferative vitreoretinopathy resulting in complex retinal re-detachment. Collagenase has been reported to liquefy the vitreous, but it has also been shown to disrupt the outer layers of the retina. Alpha-chymotrypsin has been reported to produce peripapillary and vitreous hemorrhage in the injected eyes. Finally, dispase has been reported to cause toxicity to the inner layer of the retina 15 minutes after injection. Depending on the concentration of dispase used, proliferative retinopathy or epiretinal cellular membranes can develop in the injected eyes.

Given the immunogenicity and other adverse effects of bacterial proteases, pharmacological vitreolysis using endogenous human derived proteases may be desirable. Plasmin is a serine protease derived from plasminogen. Plasminogen is an



important component of mammalian blood. Human plasminogen is a single chain glycoprotein consisting of 791 amino acids, which has a molecular weight of about 92,000 daltons (*see* Forsgren M. et al., *FEBS Lett.* **213**(2):254-60, 1987). Native plasminogen with an amino-terminal glutamic acid (termed "Glu-plasminogen") is converted by limited digestion by plasmin of the Arg<sub>68</sub>-Met<sub>69</sub>, Lys<sub>77</sub>-Lys<sub>78</sub>, or Lys<sub>78</sub>-Val<sub>79</sub> peptide bonds to proteins commonly designated as "Lys-plasminogen." Activation of plasminogen by plasminogen activators such as urokinase or streptokinase, cleaves the peptide bond between Arg<sub>561</sub> and Val<sub>562</sub> converting the plasminogen molecule into a double chain, enzymatically active form called plasmin. Plasmin contains two polypeptides, a heavy A chain connected by two disulphide bonds to a light B chain; the B chain contains the serine protease catalytic domain. The serine protease catalytic activity of plasmin has been implicated in its ability to dissolve blood clots *in vivo*.

Recently, plasmin has also been suggested as an adjunct for vitrectomy. In addition, autologous plasmin enzyme (APE) has been suggested as an agent for pharmacological vitrectomy. However, there are several disadvantages associated with the use of plasmin. First, so far all clinical interventions with plasmin have relied on the use of APE, the isolation of which necessitates a laborious and time-consuming process involving drawing of a patient's blood, isolation of plasminogen, activation of the isolated plasminogen to plasmin, and purification and sterility testing of the plasmin enzyme. Furthermore, this procedure can be costly and the presence of blood-borne pathogens can further complicate this procedure. Also, plasmin is highly prone to degradation and thus cannot be stored for prolonged periods prior to its use. A further disadvantage is plasmin's large molecular weight, which ranges between 65,000 and 83,000 daltons. Thus, the diffusion of large molecules like plasmin from its injected position in the vitreous to the vitreoretinal interface would be hindered compared to smaller molecules.

Accordingly, there is a need in the art for methods of treating or preventing disorders, or complications of disorders, of the eye of a subject that overcome the disadvantages of plasmin, for pharmacological vitreolysis. Specifically, there is a need for methods of treating or preventing a disorder, or a complication of a disorder, of the eye using smaller molecules than plasmin, which can diffuse through the vitreous to the vitreoretinal interface faster than plasmin, and which can be readily

obtained in large quantities without the delay and other attendant problems of isolating autologous plasmin enzyme on a patient-by-patient basis.

## SUMMARY OF THE INVENTION

The present invention provides methods of treating or preventing a disorder, or  
5 a complication of a disorder, of the eye of a subject using a composition comprising a truncated plasmin protein comprising a catalytic domain of plasmin (TPCD). In one embodiment, a TPCD is selected from the group consisting of miniplasmin, recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin, recombinant microplasmin,  
10 stabilized microplasmin, stabilized, recombinant microplasmin, variants of microplasmin, and any combinations thereof.

The present invention also provides methods of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject using a composition comprising a modified TPCD. A modified TPCD is a TPCD, which comprises a  
15 modified catalytic domain of plasmin.

The present invention provides methods of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject by contacting a vitreous and/or an aqueous humor of the subject with a composition comprising a TPCD. The present invention provides methods of treating or preventing eye disorders such as, but not  
20 limited to, retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia,  
25 endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa and any combinations thereof. The methods of the invention can be practiced independent of vitrectomy, or as an adjunct to vitrectomy.

The present invention also provides methods of treatment or prevention of an eye disorder, or a complication of an eye disorder, of a subject comprising  
30 administering to the subject a composition comprising at least two TPCDs. In one embodiment of this aspect of the invention, a composition is administered to a subject

by contacting a vitreous and/or an aqueous humor with a composition comprising at least two TPCDs.

5       The present invention further encompasses methods of treatment or prevention of an eye disorder, or a complication of an eye disorder, comprising providing a subject with a first composition comprising at least one TPCD, and a second composition comprising at least one TPCD. In one embodiment of this aspect of the invention, a first composition comprising at least one TPCD and a second composition comprising at least one TPCD are provided to a subject by contacting a vitreous and/or an aqueous humor. In another embodiment of this aspect of the invention, the 10 TPCDs of the first composition comprising at least one TPCD and the second composition comprising at least one TPCD are the same TPCD. In yet another embodiment of this aspect of the invention, the TPCDs of the first composition comprising at least one TPCD and the second composition comprising at least one TPCD are different TPCDs. In a further embodiment of this aspect of the invention, 15 the first composition comprising at least one TPCD and the second composition comprising at least one TPCD are administered to a subject at substantially the same time. In yet another embodiment, the first composition comprising at least one TPCD and the second compositions composition comprising at least one TPCD are administered to a subject at separate times.

20       The present invention additionally provides methods of treatment or prevention of an eye disorder, or a complication of an eye disorder, of a subject by administering a composition comprising at least one TPCD and at least one second agent to the subject. A second agent includes any substance that is useful either alone, or in combination with a TPCD, in treating or preventing an eye disorder or a 25 complication of an eye disorder. A second agent, includes without limitation, hyaluronidase, dispase, chondroitinase, collagenase, RGD containing peptides, anti-integrin antibody, urea, hydroxyurea, thiourea, P2Y receptor agonists, and any angiogenic inhibitors including, but not limited to, VEGF inhibitors and PlGF inhibitors.

30       The present invention also encompasses methods of treatment or prevention of an eye disorder, or a complication of an eye disorder, of a subject by administering to

the subject a composition comprising at least one TPCD prior to or after administration of a composition comprising a second agent.

The methods of the invention can be used to treat or prevent an eye disorder, or a complication of an eye disorder, of a subject by effecting one or more outcomes including, but not limited to, reducing the viscosity of the vitreous, liquefying the vitreous, inducing posterior vitreous detachment, clearing or reducing hemorrhagic blood from the vitreous and/or aqueous humor, clearing or reducing intraocular foreign substances from the vitreous and/or aqueous humor, clearing or reducing materials toxic to the retina, increasing diffusion of an agent or a composition administered to the vitreous and/or aqueous humor, reducing extraretinal neovascularization and any combinations thereof.

The present invention also provides methods of performing a vitrectomy comprising contacting the vitreous of a subject with a composition comprising a TPCD. The contacting step can be performed prior to or at the same time as the vitrectomy or independent of vitrectomy.

The present invention also provides a composition comprising at least two TPCDs.

The present invention further provides a composition comprising at least one TPCD and at least one second agent.

20

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 provides the DNA (SEQ ID NO:9) and amino acid sequence (SEQ ID NO:10) of human plasminogen.

FIG. 2 provides the DNA (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4) of human microplasminogen.

FIG. 3 provides the DNA (SEQ ID NO:7) and amino acid sequence (SEQ ID NO:8) of human miniplasminogen.

FIG. 4 shows the effect of treating porcine eyes with microplasmin. Panel A is a low magnification image (11X) of the mid-peripheral retina after slow dehydration of a porcine eye treated with 0.125 mg of microplasmin in 0.1 ml BSS PLUS® for 120 minutes. In the centre of this image, is a vitreous strand. It is likely that this vitreous strand is vitreous that has collapsed onto the retinal surface. Most of the retinal surface is free of vitreous as shown in the remaining panels. Panel B shows an area of bare retina adjacent to a blood vessel (magnification 800X). Few cells are seen on the retinal surface. The irregular surface is that of the vessel itself. Panel C and D are magnifications of the retinal area in B at 1200X and 3600X magnification respectively showing a smooth retinal surface largely devoid of vitreous or cellular material. At 3600x only a few fibrillar strands are visible. Panel E is an image at a magnification of 1500X essentially showing the same findings as in Panel C at a more central retinal location, while panel F shows the coarse granular structure of the vitreous, which has lost its fibrillar structure. The structure of the vitreous in the microplasmin treated eyes is very different in appearance compared to the vitreous in control eyes (data not shown).

FIG. 5 shows that ciliary processes in the porcine eye are intact after 120 minute treatment with microplasmin (Panels A and B).

FIG. 6 provides scanning electron micrographs (magnification of 3600x) of the vitreoretinal interface in human post-mortem eyes. Intravitreal injection of 62.5 µg of microplasmin resulted in posterior vitreous detachment (PVD) leaving discontinuous remnants of collagen fibrils covering the ILM (Panel A). 125 µg (Panel B) and 188 µg (Panel C) of microplasmin produced complete PVD and a bare ILM. Panel D shows the compression of collagen fibrils towards the ILM in an eye treated with 62.5 µg microplasmin and gas. Panel E shows complete PVD following treatment with 125 µg microplasmin and gas. Unlike the PVD observed in microplasmin treated eyes, there is a dense network of collagen fibrils in the control eye (Panel F).

FIG. 7 provides transmission electron micrographs of the ILM in human post-mortem eyes. Note the absence of collagen fibrils (arrows) on the ILM in the microplasmin-treated eye (Panel A, magnification 13,600X). In contrast, collagen fibrils are still present (arrows) on the ILM in the control eye (Panel B; magnification 6800X).

FIG. 8 presents scanning electron micrographs (magnification 3600X) of the vitreoretinal interface in cat eyes. Intravitreal injection of 25  $\mu$ g of microplasmin left remnants of collagen fibrils on the ILM one day after treatment (Panel A). Three days after treatment, 25  $\mu$ g of microplasmin resulted in complete PVD (Panel B).

- 5 Remnants of collagen fibrils were observed three days following treatment with 14.5  $\mu$ g of microplasmin (Panel C). A bare ILM was observed three weeks after injection of 14.5  $\mu$ g of microplasmin (Panel D) and 25  $\mu$ g of microplasmin (Panel E). In striking contrast, the control eye showed a dense attached cortical vitreous (Panel F).

- FIG. 9 presents the results of light microscopy studies of semi-thin sections of cat eyes. These studies showed that the normal cytoarchitecture of the retina observed in control eyes (Panel B) was also observed in microplasmin-treated eyes (Panel A). Transmission electron microscopy revealed a well-preserved inner retina and ILM in microplasmin-treated eyes (Panels C and E) as observed in control eyes (Panels D and F). The magnification used for Panels A and B was 250X; the magnification for  
10 Panels C and D was 6000X; while the magnification for Panels E and F was 30,000X.

- FIG.10 presents the results of confocal laser scanning microscopy with probes to glial fibrillar acidic protein (Panels A and B, green) and vimentin (Panels C and D, red). There is no difference in the staining of GFAP and vimentin between microplasmin-treated eyes (Panels A and C) and control eyes (Panels B and D).  
20 Double-label immunohistochemistry with probes to synaptophysin (green) and neurofilament (red) also shows no difference between microplasmin-treated eyes (Panel E) and a control eyes (Panel F). Magnification for Panels A,B and C was 400x; magnification for Panel D was 250X; and magnification for Panels E and F was 160x.

- FIG. 11 presents a time correlation function (TCF) of whole porcine vitreous as compared to a solution of 20 nm polystyrene nanospheres. In the vitreous there are two-components to the curve. The early (fast) component is due to the presence of hyaluronan (HA) that is freely diffusible and exhibits considerable molecular  
25 (Brownian) motion. The late (slow) component is due to collagen, which is larger and diffuses less freely (stiffer), resulting in slower Brownian movement. In contrast, the solution of polystyrene nanospheres has only one component (monodisperse) that is  
30 very fast because of the small size of the nanospheres and their perfectly spherical structure allowing for very rapid movements in the solution.

FIG. 12 presents normalized time correlation functions for 5 porcine eyes undergoing microplasmin ( $\mu$ Pli) pharmacologic vitreolysis at different doses and a solution of 20 nm polystyrene nanospheres. DLS measurements were made in the optical axis, 4 mm below the air/vitreous interface. In the untreated (vehicle) vitreous there are two-components to the curve. The early (fast) component is due to the presence of hyaluronan (HA) that is freely diffusible and exhibits considerable molecular (Brownian) motion. The late (slow) component is due to collagen, which is larger and diffuses less freely (stiffer), resulting in slower Brownian movement. At the other extreme, the solution of polystyrene nanospheres has only one component (monodisperse) that is very fast because of the small size of the nanospheres and their perfectly spherical structure allowing for very rapid movements in the solution. With increasing doses of  $\mu$ Pli there is a decrease in the slope of the TCF with disappearance of the slow component (larger molecular species) ultimately approaching the TCF of pure 20 nm nanospheres, *i.e.*, all smaller size molecular species.

FIG. 13 presents representative photographs of porcine eyes after injection of microplasmin and fluorescein. Both images are of the same eye, with the right image captured 20 minutes after the left image, demonstrating fluorescein diffusion in the vitreous.

FIG. 14 presents representative photographs of porcine eyes after injection of plasmin and fluorescein. Both images are of the same eye, with the bottom image captured 20 minutes after the top image, demonstrating fluorescein diffusion in the vitreous to a lesser degree than that seen with microplasmin-treated eyes (Fig. 13).

## DETAILED DESCRIPTION OF THE INVENTION

The patent applications, patents, and literature references cited herein indicate the knowledge of those of ordinary skill in this field and are hereby incorporated by reference in their entirety. In the case of inconsistencies between any reference cited herein and the specific teachings of the present disclosure, this disclosure will prevail.

The following detailed description and the accompanying examples are provided for purposes of describing and explaining only certain preferred embodiments of the invention, and are not intended to limit the scope of the invention

in any way. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

Prior to setting forth the invention in detail, it may be helpful to an  
5 understanding thereof to set forth definitions of certain terms that will be used hereafter.

### *Definitions*

“treating,” means the reduction or amelioration of any medical disorder to any extent, and includes, but does not require, a complete cure of the disorder.

10 “preventing” means to defend or protect against the development of a disorder, *i.e.*, to function as a prophylactic.

“disorder” means any disease, dysfunction, syndrome, condition, pain, or any combination thereof. Disorder also includes any complications from any disease, dysfunction, syndrome, condition, pain or any combination thereof.

15 “subject” means any mammal, particularly a human.

“contacting” means any mode of administration that results in interaction between a composition and an object being contacted (*e.g.*, vitreous, aqueous humor, etc.). The interaction of the composition with the object being contacted can occur at substantially the same time as the administration of the composition, over an extended  
20 period of time starting from around the time of administration of the composition, or be delayed from the time of administration of the composition.

“composition” means a combination or mixture of one or more substances.

“substance” means that which has mass and occupies space.

“foreign substance” means any substance that is determined by a medical doctor,  
25 clinician, veterinarian or researcher to be harmful or toxic to the eye of a subject and/or to be a substance that is not normally found in a healthy mammalian eye.

“ophthalmologically acceptable carrier” is a substance with which a second substance (*for e.g.*, a TPCD) can be combined, without making the second substance unsuitable



(as determined by a medical doctor, clinician, veterinarian or researcher) for its intended use in the eye of a subject. Non-limiting examples of an ophthalmologically acceptable carrier include balanced salt solution (BSS) and BSS-PLUS®.

“pharmaceutically acceptable carrier” includes, without limitation, water, buffered saline, polyol (*for e.g.*, glycerol, propylene glycol, liquid polyethylene glycol), or suitable mixtures thereof. Other examples of pharmaceutically acceptable carriers and methods for making such carriers and formulations thereof are found, for example, in Remington's Pharmaceutical Sciences (20th Edition, A. Gennaro (ed.), Lippincott, Williams & Wilkins, 2000).

10 “an effective amount” means an amount of a substance or composition that elicits a response in an eye of a human or other mammal that is being sought by a researcher, veterinarian, medical doctor or other clinician.

“inducing” means to bring about or stimulate the occurrence of a desired result.

“reduce” means to decrease to any extent.

15 “toxic effects to the eye” means any adverse effect to the eye of a subject that is determined to be harmful to the subject by a researcher, veterinarian, medical doctor or other clinician.

“vitreous” means the vitreous humor, also referred to as the vitreous body, which occupies the chamber between the crystalline lens of the eye and the retina.

20 “TPCD” is an acronym for “truncated plasmin protein comprising a catalytic domain of plasmin.” A “truncated plasmin protein” means any plasmin protein obtained by deleting one or more amino acids of Val<sub>79</sub>-plasmin (*i.e.*, amino acids 79-791 of human plasminogen), wherein the resulting protein possesses serine protease catalytic activity. Such amino acid deletions can be at the N-terminus (resulting in TPCDs  
25 consisting for example of, amino acids 444-791, 543-791, or 562-791 of SEQ ID NO:10) and/or at the C-terminus and/or at any internal position or positions of amino acids 79-791 of SEQ ID NO:10. It is to be understood that if a truncated protein derived from SEQ ID NO:10 is made as an enzymatically inactive form, it must be  
30 activated using a plasminogen activator to convert it into the corresponding active form of the truncated protein. For example, if a protein consisting of amino acids 543-

- 791 of SEQ ID NO:10 is made recombinantly, it is highly likely that the protein will not be in its enzymatically active form. Thus, the protein should be treated with a plasminogen activator to cleave the peptide bond between Arg<sub>561</sub> and Val<sub>562</sub>, thereby activating the protein. Non-limiting examples of a TPCD include miniplasmin,
- 5 recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin, recombinant microplasmin, stabilized microplasmin, stabilized, recombinant microplasmin and variants of microplasmin wherein, the variants of microplasmin and miniplasmin include a catalytic domain of plasmin.
- 10 “plasmin protein” means any protein made or derived from the amino acid sequence of human plasminogen (SEQ ID NO:10) that has a cleavage of the peptide bond between Arg<sub>561</sub> and Val<sub>562</sub> of human plasminogen. The cleavage of the peptide bond between Arg<sub>561</sub> and Val<sub>562</sub> can be accomplished using plasminogen activators. Non-limiting examples of plasmin proteins include Lys-plasmin, miniplasmin and
- 15 microplasmin.
- “catalytic domain of plasmin” means an amino acid sequence of about 130-240 amino acids derived from amino acids 543 to 791 of SEQ ID NO:10 (human plasminogen), which includes the catalytic triad of plasmin namely, His<sub>603</sub>, Asp<sub>646</sub> and Ser<sub>741</sub>, wherein the amino acid sequence possesses serine protease activity.
- 20 “modified catalytic domain of plasmin” means a catalytic domain of plasmin that has been altered by changing the amino acid sequence of the catalytic domain by addition and/or deletion and/or substitution of one or more amino acids. Of course it is to be understood that the amino acids corresponding to the catalytic triad of plasmin namely, His<sub>603</sub>, Asp<sub>646</sub> and Ser<sub>741</sub>, are not altered. The modification may increase,
- 25 decrease or leave unchanged the plasmin-like catalytic activity of the protein. For example, the modified catalytic domain of microplasmin and miniplasmin may increase, decrease or leave unchanged the catalytic activity of these proteins.
- “modified TPCD” is a TPCD containing a modified catalytic domain of plasmin, wherein the TPCD possesses plasmin-like serine protease catalytic activity.
- 30 “second agent” means any substance that can be used, either by itself, or in combination with a TPCD, in treating or preventing an eye disorder or a complication

of an eye disorder of a subject. Preferably the second agent does not prevent the catalytic activity of a TPCD.

“stabilizing a protein” means protecting a protein from degradation and/or inactivation through the use of one or more stabilizing agents.

5           Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below.

Pharmacological vitreolysis is a method of using one or more proteinaceous and/or chemical and/or nucleic acid agents to treat or prevent a disorder, or a  
10   complication of a disorder, of an eye of a subject. The present invention provides methods of pharmacological vitreolysis using at least one truncated plasmin protein comprising a catalytic domain (TPCD). Specifically, the present invention provides methods of treatment or prevention of eye disorders, or complications of eye disorders, by contacting the vitreous and/or aqueous humor with an effective amount  
15   of a composition comprising a TPCD. These methods results in outcomes such as, but not limited to, liquefaction of the vitreous, posterior vitreous detachment, reduction or clearing of hemorrhagic blood from the vitreous and/or aqueous humor, reduction or clearing of intraocular foreign substances from the vitreous and/or aqueous humor, increasing diffusion of an agent or composition administered to the vitreous and/or  
20   aqueous humor, decreasing extraretinal neovascularization, and any combinations thereof. These methods may be used either as an adjunct to vitrectomy, or in the absence of vitrectomy.

Accordingly, the present invention provides, as a first aspect, a method of treating or preventing a disorder, or a complication of a disorder, of the eye of a  
25   subject comprising contacting the vitreous and/or aqueous humor with an effective amount of a composition comprising a TPCD. In one embodiment, a TPCD has a molecular weight less than about 40,000 daltons. In another embodiment, a TPCD has a molecular weight of about 26,500 daltons in reduced form or about 29,000 daltons in non-reduced form. In yet another embodiment a TPCD has a molecular weight of  
30   between about 20,000 and 30,000 daltons. In a further embodiment, a TPCD has a molecular wight of less than about 20,000 daltons.

In a second aspect, the present invention provides a method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject comprising contacting the vitreous and/or aqueous humor with an effective amount of a composition comprising at least two TPCDs.

5 In a third aspect, the present invention provides a method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject comprising contacting the vitreous and/or aqueous humor with an effective amount of a first composition comprising at least one TPCD and an effective amount of a second composition comprising at least one TPCD. In one embodiment of this aspect of the  
10 invention, the first composition comprising at least one TPCD and the second composition comprising at least one TPCD can comprise the same TPCD. In another embodiment of this aspect of the invention, the first composition comprising at least one TPCD and the second composition comprising at least one TPCD can comprise different TPCDs. In a further embodiment of this aspect of the invention, the first and  
15 second compositions may be administered to a subject at substantially the same time or at different times.

In a fourth aspect, the present invention provides a method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject comprising contacting the vitreous and/or aqueous humor with an effective amount of  
20 a composition comprising at least one TPCD and at least one second agent. In this aspect of the invention, the second agent is not intended to be a TPCD.

In a fifth aspect, the present invention provides a method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject comprising contacting the vitreous and/or aqueous humor with an effective amount of  
25 a composition comprising at least one TPCD prior to, at the same time as, or after contacting the vitreous and/or aqueous humor with an effective amount of a composition comprising at least one second agent.

In a sixth aspect, the present invention provides a method of liquefying the vitreous comprising contacting the vitreous and/or aqueous humor with an effective  
30 amount of a composition comprising at least one TPCD. In one embodiment of this aspect of the invention, the liquefaction of the vitreous decreases the viscosity of the vitreous humor. In other embodiments of the invention, the liquefaction of the

vitreous increases the rate of clearance from the vitreous cavity and/or aqueous humor of blood, deposited material, foreign substances and/or materials toxic to the eye, especially the retina. In another embodiment of this aspect of the invention, the liquefaction of the vitreous decreases extraretinal neovascularization. In yet another embodiment of this aspect of the invention, the liquefaction of the vitreous increases the diffusion of an agent or composition administered to the vitreous and/or aqueous humor. In a further embodiment of this aspect of the invention, the liquefaction of the vitreous helps in the removal of the vitreous during standard vitrectomy or 25 Gauge (or smaller) vitrectomy.

10 In a seventh aspect, the present invention provides a method of inducing posterior vitreous detachment comprising contacting the vitreous and/or aqueous humor with an effective amount of a composition comprising at least one TPCD.

In any of the first to seventh aspects of the invention described above, the step of contacting the vitreous and/or aqueous humor with a composition comprising a TPCD can be performed as an adjunct to, or in the absence of vitrectomy.

In an eighth aspect, the present invention provides a method of performing a vitrectomy comprising the step of contacting the vitreous and/or aqueous humor with a composition comprising at least one TPCD. The contacting step can be performed at the same time as, or prior to vitrectomy.

20 In a ninth aspect, the present invention provides a composition comprising at least two TPCDs.

In a tenth aspect, the present invention provides a composition comprising at least one TPCD and at least one second agent.

In one embodiment of all aspects of the present invention, a TPCD is selected from the group consisting of miniplasmin, recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin, recombinant microplasmin, stabilized microplasmin, stabilized, recombinant microplasmin, variants of microplasmin, and any combinations thereof. In another embodiment of all aspects of the invention, the methods of treatment or prevention of an eye disorder, or complications of an eye disorder, and methods of

performing a vitrectomy result in the amelioration of an eye disorder by one or more of the following outcomes: reducing the viscosity of the vitreous, liquefying the vitreous, inducing posterior vitreous detachment, clearing or reducing hemorrhagic blood from the vitreous, vitreous cavity and/or aqueous humor, clearing or reducing intraocular foreign substances from the vitreous, vitreous cavity and/or aqueous humor, clearing or reducing materials toxic to the retina from the vitreous, vitreous cavity and/or aqueous humor, increasing the diffusion of an agent or a composition administered to the vitreous and/or aqueous humor, or reducing retinal neovascularization. In yet another embodiment of all aspects of the invention, the eye disorder or complication of an eye disorder sought to be treated or prevented is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof.

A TPCD includes any truncated plasmin protein comprising a catalytic domain of plasmin. A truncated plasmin protein encompasses any plasmin protein obtained by deleting one or more amino acids of Val<sub>79</sub>-plasmin (*i.e.*, amino acids 79-791 of human plasminogen), wherein the resulting protein possesses serine protease catalytic activity. Thus, all TPCDs must contain the catalytic triad of the plasmin serine protease domain namely, His<sub>603</sub>, Asp<sub>646</sub> and Ser<sub>741</sub>. Truncations of Val<sub>79</sub>-plasmin can be made by deletions of one or more amino acids in amino acids 79-791 of SEQ ID NO:10 (human plasminogen). Such deletions can be at the N-terminus, C-terminus or at an internal location of amino acids 79-791 of SEQ ID NO:10. It should be understood that if a protein resulting from a truncation of amino acids 79-791 of SEQ ID NO:10 (human plasminogen) is made in an enzymatically inactive form, the protein must be converted to its active form using a plasminogen activator, to be considered a TPCD. Plasminogen activators cleave the peptide bond between Arg<sub>561</sub> and Val<sub>562</sub>, thereby activating a protein. In one embodiment a TPCD includes proteins consisting essentially of amino acids 444-791 of SEQ ID NO:10. In another embodiment, a TPCD includes proteins with one or more amino acid deletions in

amino acids 444-791 of human plasminogen, wherein the resulting protein possesses serine protease catalytic activity. In another embodiment, a TPCD includes proteins consisting essentially of amino acids 543-791 of SEQ ID NO:10. In yet another embodiment a TPCD includes proteins with one or more deletions in amino acids 543-791 of human plasminogen, wherein the resulting protein possesses serine protease catalytic activity. In another embodiment, a TPCD includes proteins consisting essentially of amino acids 562-791 of SEQ ID NO:10. In another embodiment a TPCD includes proteins with one or more deletions in amino acids 562-791 of human plasminogen, wherein the resulting protein possesses serine protease catalytic activity. The deletions can be at the N-terminus, C-terminus or at an internal location of amino acids 444-791, 543-791 and 562-791 of SEQ ID NO:10 (human plasminogen), respectively. Methods of making amino acid deletions in a protein are well known to those of ordinary skill in the art (*for e.g.*, Current Protocols in Molecular Biology, Ausubel et al. (eds.), John Wiley & Sons, 2001; and Molecular Cloning: A Laboratory Manual, Third Edition, Sambrook and Russell, 2000). The catalytic activity of a TPCD can be determined by measuring the amidolytic activity of the TPCD, using the chromogenic substrate S2403 (Chromogenix, Antwerp, Belgium) (*see* Example 2), any other chromogenic substrate, or by any other methods known in the art.

The present invention also envisions the use of a modified TPCD. A modified TPCD is a TPCD with a modified form of the catalytic domain of plasmin, wherein the modified TPCD possesses plasmin-like serine protease catalytic activity. Modifications to the catalytic domain include amino acid insertions and/or deletions and/or substitutions in the catalytic domain of plasmin. However, the amino acids corresponding to the catalytic triad of the plasmin serine protease domain namely, Histidine<sub>603</sub>, Aspartic Acid<sub>646</sub> and Serine<sub>741</sub> are not altered. Preferably the modifications of the catalytic domain involve one or more conservative substitution(s) of amino acids that are not part of the catalytic triad. Conservative amino acid substitutions and methods of making such conservative amino acid substitutions are well known to one of ordinary skill in the art (*see, e.g.*, Current Protocols in Molecular Biology, *supra*; and Molecular Cloning: A Laboratory Manual, *supra*). These modifications may increase, decrease or leave unchanged the catalytic activity of the original domain. The catalytic activity of a modified TPCD can be determined by measuring the amidolytic activity of the TPCD, using the chromogenic substrate

S2403 (Chromogenix, Antwerp, Belgium) (see Example 2) or by any other methods known in the art.

TPCD includes, but is not limited to, miniplasmin, recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin, recombinant microplasmin, stabilized microplasmin, stabilized, recombinant microplasmin, and variants of microplasmin. Variants of microplasmin and miniplasmin include shorter forms of microplasmin and miniplasmin that can be produced by amino acid deletions from these proteins. All variants of microplasmin and miniplasmin are expected to have serine protease catalytic activity, even if they do not possess the same level of catalytic activity as microplasmin and miniplasmin, respectively. Thus, all variants of microplasmin and miniplasmin are required to contain amino acids 603-741 of SEQ ID NO:10, which contains the catalytic triad of the plasmin serine protease domain namely, His<sub>603</sub>, Asp<sub>646</sub> and Ser<sub>741</sub> of human plasminogen. In one embodiment a variant of miniplasmin includes proteins containing one or more amino acid deletions in amino acids 444-791 of human plasminogen, wherein the resulting protein possesses serine protease catalytic activity. In another embodiment, a variant of microplasmin includes proteins containing one or more deletions in amino acids 543-791 of human plasminogen, wherein the resulting protein possesses serine protease catalytic activity. In yet another embodiment, a variant of microplasmin includes proteins containing one or more deletions in amino acids 562-791 of human plasminogen, wherein the resulting protein possesses serine protease catalytic activity. The deletions can be at the N-terminus, C-terminus or at an internal location of amino acids 444-791, 543-791 and 562-791 of SEQ ID NO:10, respectively; however, all variants of microplasmin and miniplasmin are required to contain amino acids 603-741 of SEQ ID NO:10. Variants of microplasmin and miniplasmin also include, but are not limited to, amino acid insertions and/or substitutions in these proteins. It is envisioned that amino acid substitutions made in microplasmin or miniplasmin are preferably conservative substitutions. Any variant of microplasmin and miniplasmin or any other TPCD can be prepared by recombinant methods and activated to the active plasmin form with a plasminogen activator. Alternatively, variants of microplasmin and miniplasmin or any other TPCD can be prepared by any other means well known in the art such as, but not limited to, digestion of human plasminogen with elastase or partial reduction and alkylation of



plasmin, microplasmin or miniplasmin. These variants of microplasmin, miniplasmin, or for that matter any TPCD, can be assayed for serine protease catalytic activity using the chromogenic substrate S2403 or any other chromogenic substrate. In addition, the variants of microplasmin, miniplasmin or any other TPCD can be tested for their

5 ability to induce PVD and/or effect vitreous liquefaction by injecting different doses of the variant in any balanced saline solution into porcine, feline or post-mortem human eyes. If a TPCD can induce PVD and/or effect vitreous liquefaction in any of these eyes, that TPCD is considered to be useful for treating eye disorders of mammals. Preferably, the TPCD does not result in toxicity to the injected eye. Non-

10 limiting examples of variants of microplasmin are provided in Table 1.

**TABLE 1****Non-limiting Examples of Variants of Microplasmin**

The variants of microplasmin listed below correspond to the amino acid sequence and numbering of human plasminogen, which consists of amino acids 1-791

15 (see, Fig.1, SEQ ID NO:10).

542-741, 542-742, 542-743, 542-744, 542-745, 542-746, 542-747, 542-748, 542-749, 542-750, 542-751, 542-752, 542-753, 542-754, 542-755, 542-756, 542-757, 542-758, 542-759, 542-760, 542-761, 542-762, 542-763, 542-764, 542-765, 542-766, 542-767, 542-768, 542-769, 542-770, 542-771, 542-772, 542-773, 542-774, 542-775, 542-776,

20 542-777, 542-778, 542-779, 542-780, 542-781, 542-782, 542-783, 542-784, 542-785, 542-786, 542-787, 542-788, 542-789, 542-790, 542-791;

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25 543-768, 543-769, 543-770, 543-771, 543-772, 543-773, 543-774, 543-775, 543-776, 543-777, 543-778, 543-779, 543-780, 543-781, 543-782, 543-783, 543-784, 543-785, 543-786, 543-787, 543-788, 543-789, 543-790, 543-791;

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545-741, 545-742, 545-743, 545-744, 545-745, 545-746, 545-747, 545-748, 545-749, 545-750, 545-751, 545-752, 545-753, 545-754, 545-755, 545-756, 545-757, 545-758, 545-759, 545-760, 545-761, 545-762, 545-763, 545-764, 545-765, 545-766, 545-767,

35 545-768, 545-769, 545-770, 545-771, 545-772, 545-773, 545-774, 545-775, 545-776,

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- 546-741, 546-742, 546-743, 546-744, 546-745, 546-746, 546-747, 546-748, 546-749, 546-750, 546-751, 546-752, 546-753, 546-754, 546-755, 546-756, 546-757, 546-758, 546-759, 546-760, 546-761, 546-762, 546-763, 546-764, 546-765, 546-766, 546-767, 546-768, 546-769, 546-770, 546-771, 546-772, 546-773, 546-774, 546-775, 546-776, 546-777, 546-778, 546-779, 546-780, 546-781, 546-782, 546-783, 546-784, 546-785, 546-786, 546-787, 546-788, 546-789, 546-790, 546-791;
- 547-741, 547-742, 547-743, 547-744, 547-745, 547-746, 547-747, 547-748, 547-749, 547-750, 547-751, 547-752, 547-753, 547-754, 547-755, 547-756, 547-757, 547-758, 547-759, 547-760, 547-761, 547-762, 547-763, 547-764, 547-765, 547-766, 547-767, 547-768, 547-769, 547-770, 547-771, 547-772, 547-773, 547-774, 547-775, 547-776, 547-777, 547-778, 547-779, 547-780, 547-781, 547-782, 547-783, 547-784, 547-785, 547-786, 547-787, 547-788, 547-789, 547-790, 547-791;
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Miniplasmin and microplasmin are produced upon the activation of miniplasminogen and microplasminogen by plasminogen activators such as, but not limited to, streptokinase, staphylokinase, tissue-type plasminogen activator or urokinase. Miniplasminogen and microplasminogen are derived from plasminogen, which is a single chain glycoprotein that is an important component of mammalian blood. Human plasminogen is a multi-domain protein of 791 residues (SEQ ID NO:10), composed of an N-terminal pre-activation domain, five homologous kringle domains each of about 80 amino acids, a serine protease catalytic domain and inter-domain connecting sequences. Plasmin or plasminogen activators cleave the peptide bonds between Arg<sub>68</sub>-Met<sub>69</sub>, or Lys<sub>77</sub>-Lys<sub>78</sub> or Lys<sub>78</sub>-Val<sub>79</sub> at the N-terminal of human plasminogen, resulting in shorter proenzymes called Lys-plasminogens (for example,

proteins consisting of amino acids 69-791 or 78-791 or 79-791). Additional cleavage by the enzyme elastase removes the first four kringle domains producing the proenzyme, miniplasminogen (typically amino acids 442-791). Further cleavage of the fifth kringle yields the proenzyme, microplasminogen (typically amino acids 543-791). The kringles of plasminogen contain lysine-binding sites that mediate specific binding of plasminogen to substrates such as fibrin. The proenzyme forms of plasminogen are activated to their enzymatically active form by the cleavage of the peptide bond between Arg<sub>561</sub> and Val<sub>562</sub> to yield a disulfide bonded double chain form of the corresponding protein. The product of activation of a plasminogen protein is called a plasmin. Thus, the product of Lys-plasminogen activation is called Lys-plasmin, while the products of activation of miniplasminogen and microplasminogen, are referred to as miniplasmin and microplasmin, respectively. Lys-plasmin has a molecular weight of about 65,000 in its unglycosylated form and a molecular weight of about 83,000 daltons in its fully glycosylated form, while miniplasmin has a molecular weight of about 38,000 daltons, and microplasmin has a molecular weight of about 26,500 daltons in the reduced form and about 29,000 daltons in the non-reduced form. Like plasmin, miniplasmin and microplasmin possess catalytic activity. An advantage of miniplasmin and microplasmin over plasmin is their smaller size compared to plasmin. Thus, both microplasmin and miniplasmin are expected to have faster diffusion rates in the vitreous than plasmin (Xu, J. et al., *Pharmaceutical Research* 17: 664-669, 2000).

In one embodiment, a TPCD has a molecular weight of less than about 40,000 daltons. In another embodiment, a TPCD has a molecular weight of between about 20,000 and about 30,000 daltons. In yet another embodiment, a TPCD has a molecular weight of about 26,500 daltons in reduced form and about 29,000 daltons in non-reduced form. In a further embodiment a TPCD has a molecular weight less than about 20,000 daltons.

Microplasmin can be prepared by the autolytic reaction of plasmin and plasminogen in high alkaline solution having a pH ranging from about 9.5 to 11.5, as described in U.S. Patent No. 4,774,087. Alternatively, microplasmin and miniplasmin can be prepared by recombinant methods as described in PCT application WO 02/50290. Briefly, DNA encoding miniplasminogen and microplasminogen are independently cloned into a yeast expression vector (*for e.g.*, pPICZa A secretion

vector from Invitrogen Corporation) that can be used to express these proteins in methylotropic yeasts (*e.g.*, *Hansenula*, *Pichia*, *Candida*, and *Torulopsis*). Yeast clones that produce proteins with the highest miniplasmin and microplasmin activity are selected for large-scale production. These clones can be grown at any scale, but typically at about a 20 liter to about a 500 liter scale. The secreted miniplasminogen or microplasminogen are purified in a three-step process comprising cation exchange expanded bed chromatography, hydrophobic chromatography, and affinity chromatography. The purified microplasminogen and miniplasminogen obtained by this process are activated to their active forms using a molar ratio of a plasminogen activator (*e.g.*, urokinase, streptokinase, staphylokinase, the SY162 staphylokinase variant, etc.). It should be noted that the recombinant process for producing miniplasmin and microplasmin can be extended to produce any TPCD. An advantage of using a recombinant TPCD compared to autologous plasmin enzyme is that the recombinant proteins can be prepared from large production batches resulting in enzymes of uniform activity. Because these proteins are of uniform activity, standardized protocols can be implemented. A further advantage is that these proteins could be readily available without the delay and other attendant problems associated with the isolation and purification of plasmin from each patient.

The TPCD obtained by the processes described above can be concentrated, stabilized and/or lyophilized. Methods of concentrating proteins are well known to those of ordinary skill in the art (*see* for example, Protein Purification Methods: A Practical Approach, Harris, E.L.V and Angal, S. (eds.), IRL Press, 1989; A Guide to Protein Isolation (Second Edition), Clive Dennison, Kluwer Academic Publications, 2003; and Protein Methods (Second Edition), Daniel M. Bollag, Michael D. Rozycki, Stuart J. Edelstein (eds.), Wiley, 1996).

Stabilization is a method of protecting a protein from degradation and/or inactivation through the use of one or more stabilizing agents (*for e.g.*, by contacting a TPCD with a stabilizing agent, or purifying a TPCD in the presence of a stabilizing agent). Stabilizing agents include without limitation, tranexamic acid, hexanoic acid, lysine, serine, threonine, methionine, glutamine, alanine, glycine, isoleucine, valine, alanine aspartic acid, polyhydric alcohol, pharmaceutically acceptable carbohydrates, glucosamine, thiamine, niacinamide, any acidic buffer comprising citric acid, acetic acid, hydrochloric acid, carboxylic acid, lactic acid, malic acid, tartaric acid, or

benzoic acid, and salts such as sodium chloride, potassium chloride, magnesium chloride, calcium chloride, and any derivatives or combinations thereof. One advantage of using stabilized, recombinantly produced TPCD compared to autologous plasmin enzyme is that these proteins are more stable than autologous plasmin enzyme, which is obtained by collecting blood and, purifying, preparing and storing plasmin enzyme on a patient-by-patient basis. Unlike autologous plasmin enzyme, which needs to be used very soon after its preparation, stabilized, recombinant TPCD can be used even after a significant period of time from the time of purification.

Lyophilization of a TPCD of the invention can be performed immediately after the concentration of the purified proteins or after stabilization. Methods of lyophilizing proteins are well known to those of ordinary skill in the art. The lyophilized TPCD can be stored in vials (*e.g.*, glass) in any amount, but preferably, in amounts that can be readily reconstituted for use.

Lyophilized microplasmin, miniplasmin, or any other TPCD, can be reconstituted in an ophthalmologically acceptable carrier prior to being used for contacting the vitreous and/or aqueous humor. In one embodiment an ophthalmologically acceptable carrier is a sterile solvent having a pH and osmolarity that is compatible with the vitreous of the subject. Nonlimiting examples of ophthalmologically acceptable carriers are isotonic saline solution, balanced salt solution (BSS) and BSS PLUS®. A balanced salt solution typically contains: 0.64% sodium chloride, 0.075% potassium chloride, 0.048% calcium chloride dehydrate, 0.03% magnesium chloride hexahydrate, 0.39% sodium acetate trihydrate, 0.17% sodium citrate dihydrate, sodium hydride/hydrochloric acid to adjust the pH, and water.

The method of contacting the vitreous and/or aqueous humor using compositions comprising a TPCD will depend upon the particular subject, the severity of the condition being treated and the dosage required for therapeutic efficacy, and can be determined by a physician on a patient-by-patient basis. Any method of contacting the vitreous and/or aqueous humor that provides an effective amount of a TPCD to the vitreous and/or aqueous humor can be utilized. It should be understood that such contact with the vitreous and/or aqueous humor does not have to take place simultaneously with the administration of a composition comprising a TPCD. The

contact may be delayed or occur over an extended period of time from the time of administration. One method of contacting the vitreous and/or aqueous humor is by one or more intraocular injections directly into the vitreous and/or aqueous humor respectively. The vitreous and/or aqueous humor can also be contacted by sub-  
5 conjunctival, intramuscular or intravenous injections. Any of these injections can be provided using a liquid solution comprising a TPCD according to procedures well known in the art. Alternatively, however, the vitreous and/or aqueous humor can be contacted with a TPCD by any other suitable method, which results in sufficient distribution of the TPCD to the vitreous and/or aqueous humor to treat or prevent the  
10 disorder, or a complication of a disorder, of the eye of a subject. A composition comprising a TPCD can also be administered by placing an intra-vitreous implantable devices including, but not limited to, OCUSER<sup>®</sup> (Alza Corp., Palo Alto, Calif.) and VITRASERT<sup>®</sup> (Bausch and Lomb, Inc., Rochester, N.Y.). The present invention also envisions that the vitreous and/or aqueous humor can be contacted with a TPCD  
15 using a depot, sustained release formulation, or any implantable device so that a TPCD is supplied continuously.

Dosing regimens for TPCD can be readily determined by one of ordinary skill in the art and will vary depending on the patient and the effect sought. TPCD can be used at any dose, which brings about desirable therapeutic effects, including but not  
20 limited to vitreous liquefaction, posterior vitreous detachment, and/or clearing of blood, toxic materials or foreign substances from the vitreous cavity, without causing significant toxicity to the eye (especially the retina) or associated anatomical structures. Additionally, a TPCD may be administered as a single dose or in multiple doses. A typical TPCD dosage is in the range of about 0.005 mg to about 0.2 mg per  
25 eye. If injected, TPCD can be provided in a delivery volume of about 0.05 ml to about 0.3 ml of a sterile solvent (*e.g.* sterile BSS or BSS PLUS<sup>®</sup>) per eye. In those instances where a vitrectomy is to be performed, the TPCD is left in the vitreous and/or aqueous humor for between about 15 and 120 minutes before removal of the vitreous. In one embodiment of the invention, a dose of 0.125 mg of TPCD is  
30 delivered in 0.1 ml of sterile BSS or BSS PLUS<sup>®</sup> per eye. In another embodiment, a dose of 0.125 mg of TPCD is delivered in 0.1 ml of sterile BSS or BSS PLUS<sup>®</sup> per eye for about 15-120 minutes prior to vitrectomy.

The present invention also contemplates the use of compositions comprising more than one TPCD. Accordingly, in one aspect of the invention, the vitreous and/or aqueous humor is contacted with a composition comprising a first TPCD and a second TPCD. In one particular embodiment of this aspect of the invention, the first and  
5 second TPCD are selected from the group consisting of miniplasmin, recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin, recombinant microplasmin, stabilized microplasmin, stabilized, recombinant microplasmin, variants of microplasmin, and any combinations thereof. In another aspect of the invention, the vitreous and/or aqueous  
10 humor is contacted with a first composition comprising at least one TPCD and with a second composition comprising at least one TPCD. The TPCD can be the same or different proteins and can be administered at substantially the same time or at different times. Additionally, a TPCD can also be administered as a composition further comprising at least one second agent. Furthermore, the vitreous and/or aqueous  
15 humor may be contacted with a composition comprising at least one TPCD followed by a composition comprising at least one second agent or *vice versa*. This may be necessary where the time required for each of these compositions is different, i.e., where one compositions needs more time to act compared to the other. A second agent is any protein (but not a TPCD), chemical or other substance that is useful in  
20 treating or preventing eye disorders, or complications of an eye disorder. Such second agents are described in U.S. Patent Nos.: 4,820,516; 5,292,509; 5,866,120; 6,051,698; 6,462,071; 6,596,725; and 6,610,292. Non-limiting examples of second agents usable with the present invention include glycosaminoglycanase enzymes such as hyaluronidases, chondroitinase ABC, chondroitinase AC, chondroitinase B,  
25 chondroitin 4-sulfatase, chondroitin 6-sulfatase and B-glucuronidase; collagenase enzymes; dispase; RGD containing peptides such as RGD, GRGDS, GRGDTP, Echistatin and Falvoridin; anti-integrin antibody; P2Y receptor antagonists; urea, hydroxyurea, thiourea and anti-angiogenic agents such as, but not limited to, vascular endothelial growth factor (VEGF) inhibitors (*e.g.*, anti-VEGF antibodies, VEGF  
30 aptamers, soluble VEGF receptors, etc.) and placental growth factor (PlGF) inhibitors (*e.g.*, anti-PlGF antibodies, PlGF aptamers, soluble VEGF receptors, etc.). Most of these second agents are themselves capable of promoting vitreous liquefaction and/or inducing posterior vitreous detachment. Anti-angiogenic second agents could be useful in preventing neo-vascularization in the eye. Expression of VEGF and/or PlGF

from an hypoxic retina are thought to result in the development of extraretinal neovascularization. Thus, inhibiting VEGF and/or PlGF would be an effective way to prevent neovascularization.

A composition comprising a TPCD is useful to effect the liquefaction of the vitreous and/or the disinsertion or detachment of the vitreous from the retina and other tissues (*e.g.*, epiretinal membranes, macula). As a result of this vitreous liquefaction and/or vitreous detachment, the tractional forces of the vitreous on the retina and other tissues are minimized and the rate of natural turnover of fluids within the vitreous is accelerated. Accordingly, compositions comprising a TPCD are particularly suitable for the treatment or prevention of many disorders of the eye, which benefit from vitreous liquefaction, posterior vitreous detachment, decreasing extraretinal neovascularization and/or accelerated clearance of toxins or other deleterious substances (*e.g.*, angiogenic factors, edema fluids, hemorrhagic blood etc.) from the posterior chamber of the eye and/or tissues adjacent to the posterior chamber (*e.g.*, retina or macula). Examples of such eye disorders include, but are not limited to, retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma and retinitis pigmentosa, and others in which the clinical symptoms of these disorders respond to TPCD administration. The present invention contemplates the treatment of disorders of the eye comprising contacting the vitreous with a composition comprising a TPCD. Such contact is expected to liquefy the vitreous and/or induce posterior vitreous detachment and/or clear the vitreous cavity of blood or other toxic substances and/or decrease extraretinal neovascularization, thereby treating or preventing the disorder.

The present invention is also directed to methods of preventing or inhibiting the onset of various disorders of the eye that are the result of, or exacerbated by, vitreous adhesion to the retina and vitreous contraction. In one embodiment, the methods of the present invention are able to prevent or inhibit the disorders, or complications resulting from a disorder in the eye of a subject without removing the vitreous from the eye. In particular, the invention is directed to a process of treating a

patient with proliferative disorders or at risk of developing proliferative disorders, such as, but not limited to, a diabetic patient, by inducing posterior vitreous detachment as a prophylactic step in preventing or delaying the onset of disorders associated with vitreous contraction or neovascularization into the vitreous. In one  
5 embodiment of the invention, the composition is introduced into the eye of a diabetic patient to inhibit progression of diabetic retinopathy. Preferably, the composition is introduced into the eye before the proliferative disorders occur. In one embodiment, the composition is introduced into the vitreous of the eye before the onset of  
10 proliferative disorders and allowed to remain in the eye indefinitely without removing the vitreous from the eye. In further embodiments, the invention is directed to a process for inhibiting complications in central and branch retinal vein occlusion, such as retinal neovascularization and macular edema by inducing posterior vitreous detachment in a patient in need of such treatment. The present invention provides a process for treating impending or full-thickness macular hole (whether idiopathic or  
15 traumatic) by inducing posterior vitreous detachment. Preventing or reducing the incidence of retinal detachment, retinal tears and retinal hemorrhage caused by vitreous contraction can be achieved by inducing posterior vitreous detachment before such disorders occur and without removing the vitreous from the eye.

Many ophthalmic disorders have as a causative component, a destabilization of  
20 the blood-retina membrane. This destabilization permits various components (*e.g.*, serum components, lipids, proteins) of the choriocapillaries to enter the vitreal chamber and damage the retinal surface. This destabilization is also a precursor to vascular infiltration of the vitreal chamber, known as neovascularization. Neovascularization of the vitreous is dependent on the matrix of the vitreous. Thus,  
25 liquefaction of the vitreous, which removes the matrix in the form of the polymerized vitreous, blocks neovascularization. In one embodiment, the invention provides a method of treating or preventing eye disorders by preventing or reducing the incidence of retinal neovascularization comprising contacting the vitreous with a composition comprising a TPCD.

30 Several ophthalmological disorders including diabetic retinopathy and trauma result in the rupture or leakage of retinal blood vessels with resultant bleeding into the vitreous (*i.e.*, vitreous hemorrhage). Vitreous hemorrhage typically manifests as clouding or opacification of the vitreous and is sometimes, but not always,



accompanied by tearing or detachment of the retina. In cases where the vitreous hemorrhage is accompanied by a retinal tear or detachment, it is important that such retinal tear or detachment be promptly diagnosed and surgically repaired. Failure to promptly diagnose and repair the retinal tear or detachment may allow photoreceptor cells of the retina, in the region of the tear or detachment, to become necrotic. Necrosis of the photoreceptor cells of the retina may result in loss of vision. Furthermore, allowing the retinal detachment to remain unrepaired for such extended period of time may result in further vitreous hemorrhage and/or the formation of fibrous tissue at the site of the hemorrhage. Fibrous tissue may result in the formation of an undesirable permanent fibrous attachment between the vitreous body and the retina. In the absence of any treatment, hemorrhagic clouding of the vitreous can take between 6-12 months or longer to clear sufficiently to allow trans-vitreous viewing of the retina. In such cases, where a physician would need to repair any part of the retinal surface, or where a physician would need to view the retinal surface of a patient that is prevented by an opaque or cloudy vitreous, a microsurgical procedure known as vitrectomy may need to be performed. This procedure involves removal of all or a portion of the vitreous with a microsurgical cutter and the replacement of the vitreous with a clear liquid or other substance that allows the ocular cavity to maintain its shape. Standard vitrectomy surgical procedures are well known to those of ordinary skill in the art. In one embodiment, the present invention contemplates contacting the vitreous with a composition comprising at least one TPCD as an adjunct to vitrectomy. In other embodiments, the vitreous is contacted with the composition comprising at least one TPCD in the absence of performing a vitrectomy.

The invention is illustrated further by the following examples, which are not to be construed as limiting the invention in scope or spirit to the specific procedures described therein. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

## EXAMPLE 1

Vector construction for expression of human microplasminogen and human miniplasminogen in *Pichia pastoris*(a) *The pPICZ $\alpha$ A vector*

5           The pPICZ $\alpha$  A secretion vector purchased from Invitrogen Corporation (Carlsbad, California) was used to direct expression and secretion of recombinant human microplasminogen and miniplasminogen in *Pichia pastoris*. Notable features of this vector include: (i) a 942 bp fragment containing the alcohol oxidase 1 (*AOX1*) promoter that allows methanol-inducible, high level expression of recombinant protein  
10 in *Pichia*, as well as targeted plasmid integration to the *AOX1* chromosomal locus; (ii) the native transcription termination and polyadenylation signal from the *AOX1* gene; (iii) an expression cassette conferring zeocin resistance to *Escherichia coli* and *Pichia pastoris*; (iv) a ColE1 origin of replication for propagation and maintenance of the plasmid in *Escherichia coli*; (v) a c-myc epitope and a polyhistidine (6X His) tag,  
15 which can be used for protein detection and purification; and (vi) unique restriction sites (for example, Sac I, Pme I, BstXI) that permit linearization of the vector at the *AOX1* locus for efficient integration into the *Pichia* genome.

In addition to the above features, this vector contains the secretion signal of the *Saccharomyces cerevisiae*  $\alpha$ -factor prepropeptide, allowing expression of  
20 heterologous proteins as secreted proteins into the medium. The processing of the  $\alpha$  factor mating signal sequence in pPICZ $\alpha$  occurs in two steps:

1.       the preliminary cleavage of the signal sequence by the *KEX2* gene product occurring between arginine and glutamine in the sequence Glu-Lys-Arg \* Glu-Ala-Glu-Ala, where \* is the site of cleavage. However, the Glu-Ala repeats are not always  
25 necessary for cleavage by Kex2.
2.       the Glu-Ala repeats are further cleaved by the *STE13* gene product. In some cases where Ste13 cleavage is not efficient, the Glu-Ala repeats are left on the NH<sub>2</sub>-terminus of the expressed protein of interest.

Engineered immediately downstream of the  $\alpha$  factor signal sequence in the pPICZ $\alpha$  A vector is a multi-cloning site with recognition sequences for the enzymes EcoR I, Sfi I, Kpn I, Xho I, Sac II and Xba I to facilitate the cloning of foreign genes. In addition to the Xho I site in the multiple cloning site, there is a Xho I recognition sequence at the carboxyl-terminus of the  $\alpha$  factor secretion signal, immediately upstream of the Lys-Arg Kex2 cleavage site. This Xho I restriction site may be used to clone the gene of interest flush with the Kex2 cleavage site by using a PCR cloning approach and an appropriate forward primer to rebuild the sequence from the Xho I site to the arginine codon. The recombinant protein of interest will then be expressed with a native NH<sub>2</sub>-terminus.

(b) *Expression vector construction for microplasminogen*

The nucleic acid sequence encoding the human microplasminogen protein (amino acids 543 to 791 (SEQ ID NO: 4)) was amplified ("PCR-rescue") from the vector Fmyc- $\mu$ Pli (Lasters et al. in *Eur. J. Biochem.* **244**:946, 1997) using the Advantage cDNA polymerase mix available from Clontech (Palo Alto, California). After a DNA template denaturation step of 3 minutes at 94°C, 30 rounds of thermal cycling were performed (30 seconds at 94°C, 30 seconds at 50°C, 30 seconds at 72°C), followed by a 2 minutes final elongation step at 72°C. The following oligonucleotide primers LY-MPLG1 (sense) and LY-MPLG2 (antisense) were used in this reaction:

LY-MPLG1: 5' GGGGTATCT CTC GAG AAA AGA GCC CCT TCA TTT GAT TG  
(SEQ ID NO:1)

LY-MPLG2: 5' GTTTTGT TCT AGA TTA ATT ATT TCT CAT CAC TCC CTC  
(SEQ ID NO: 2)

The LY-MPLG1 primer had an annealing region corresponding to residues 543-548 of human plasminogen (Ala-Pro-Ser-Phe-Asp-Cys) preceded by a non-annealing extension which included the last four residues of the  $\alpha$  factor mating signal (Leu-Glu-Lys Arg). In this extension, the Leu-Glu codons determine the Xho I restriction site (underlined) allowing the cloning of the gene of interest flush with the Kex2 cleavage site. The LY-MPLG2 primer had an annealing region corresponding

to the last seven residues of plasminogen, followed by a TAA stop-codon and a non-annealing region comprising a Xba I recognition sequence (underlined).

The amplified fragment having the expected size (~ 780 bp) was digested with Xho I and Xba I, and directionally cloned into the vector pPICZ $\alpha$  A. The recipient  
5 vector-fragment was prepared by Xho I and Xba I restriction, and purified from agarose gel using the Qiaquick gel extraction kit (Qiagen GmbH, Germany). The *E.coli* strain TG1 (DSMZ collection #1208, Germany) was transformed with the ligation mixture, and zeocin resistant clones were selected. Based on restriction analysis, a plasmid clone containing an insert of the expected size was retained for  
10 further characterization. Sequence determination of the vector pPICZ $\alpha$ -MPLG1 (clone #5) using the primers 5'AOX and 3'AOX, which were provided in the EasySelect *Pichia* expression kit from Invitrogen Corporation (Carlsbad, California), confirmed the precise insertion of the microplasminogen coding region fused to the  $\alpha$  factor mating signal, as well as the absence of unwanted mutations in the coding  
15 region.

The determined nucleotide sequence and the deduced amino-acid sequence of human microplasminogen used are represented in SEQ ID NO: 3 and SEQ ID NO: 4, respectively. Compared to the sequence previously determined by Forsgren et al. in *FEBS Lett.* **213**: 254, 1987, the nucleotide sequence differs in 10 positions. However,  
20 the amino acid sequence was identical.

(c) *Expression vector construction for miniplasminogen*

A pPICZ $\alpha$ -derived secretion vector was constructed as follows for miniplasminogen expression, making use of the hereinabove described pPICZ $\alpha$ -MPLG1 vector.

25 A 500 bp DNA fragment encoding kringle five and part of the catalytic domain of the miniplasminogen protein (amino acids 444 to 604 of SEQ ID NO:10) was amplified ("PCR-rescue") from the vector FdTet-SN-miniPlg (Lasters et al., cited *supra*). After a DNA template denaturation step of 3 minutes at 94°C, 30 rounds of thermal cycling were performed (10 seconds at 94°C, 10 seconds at 50°C, 15 seconds  
30 at 72°C), followed by a 2 minutes final elongation step at 72°C. The following

oligonucleotide primers LY-MINPLG1 (sense) and LY-MINPLG2 (antisense) were used in this reaction:

LY-MINPLG1: 5' GGGGTATCT CTC GAG AAA AGA GCA CCT CCG CCT GTT  
GTC CTG CTT CC (SEQ ID NO: 5)

5 LY-MINPLG2: 5' GCA GTG GGC TGC AGT CAA CAC CCA CTC (SEQ ID NO: 6)

The LY-MINPLG1 primer has an annealing region corresponding to residues 444-452 of plasminogen (Ala-Pro-Pro-Pro-Val-Val-Leu-Leu-Pro) preceded by a non-annealing extension which included the last four residues of the factor mating signal (Leu-Glu-Lys-Arg). In this extension, the Leu-Glu codons determine the Xho I  
10 restriction site (underlined) allowing the cloning of the gene of interest flush with the Kex2 cleavage site.

The LY-MINPLG2 primer has an annealing region corresponding to the residues 596-604 of human plasminogen (Glu-Trp-Val-Leu-Thr-Ala-Ala-His-Cys).  
15 This annealing region of the catalytic domain, also present in the microplasminogen expression vector, comprises a unique Pst I recognition sequence (underlined).

The amplified fragment having the expected size was digested with Xho I and Pst I, and directionally cloned into the recipient vector fragment derived from pPICZ $\alpha$ -MPLG1 (described above). The recipient vector-fragment was prepared by  
20 Xho I and Pst I restriction, and purified from agarose gel using the Qiaquick gel extraction kit (Qiagen GmbH, Germany). The *E. coli* strain TG1 (DSMZ collection #1208, Germany) was transformed with the ligation mixture, and zeocin resistant clones were selected. Based on restriction analysis, a plasmid clone containing an insert of the expected size was retained for further characterization. Sequence  
25 determination of the vector pPICZ $\alpha$ -KMPLG1 (clone #3), using the primers 5'AOX and 3'AOX, confirmed the precise insertion of the amplified fragment fused to the  $\alpha$ -factor mating signal, as well as the absence of any mutations in the cloned region.

## EXAMPLE 2

## Method of Preparing Recombinant, Stabilized Microplasmin

(a) *Transformation of Pichia with pPICZ $\alpha$ -MPLG1*

Ten  $\mu$ g of the vector pPICZ $\alpha$ -MPLG1 was digested with Pme I, which  
5 linearizes the vector in the 5' AOX1 region. The DNA was precipitated and  
concentrated to about 0.33  $\mu$ g/ $\mu$ l in sterile distilled water, and 5  $\mu$ l was used to  
transform competent *Pichia pastoris* X33 cells prepared according to the manual  
provided in the EasySelect Pichia expression kit.

(b) *Selection of a High-Expression Strain*

10 The selection of a high-expression strain was performed as follows. Zeocin  
resistant transformants were selected on YPDSZ plates (1% yeast extract, 2% peptone,  
2% glucose, 1M sorbitol, 2% agar, 100  $\mu$ g/ml zeocin). Thirty-four single colonies  
were inoculated in 10 ml BMYZ-glycerol medium (1% yeast extract, 2% peptone, 1%  
glycerol, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base,  $4 \times 10^{-5}$  %  
15 biotin, 100  $\mu$ g/ml zeocin) in 50 ml Falcon tubes and cultured for 16 hours at 30°C.  
The cells were pelleted and re-suspended in 2 ml of BMYZ-methanol medium (same  
as BMYZ-glycerol but with 0.5% methanol instead of glycerol) to induce expression  
from the AOX1 promoter, and cultured for 40 hours. 4 pulses of 0.5% methanol were  
supplied to the cultures over this period (after 6, 22, 26 and 30 hours). At the end of  
20 the induction culture, the presence of microplasminogen in the culture supernatant was  
estimated as described by Lijnen et al. in *Eur. J. Biochem.* **120**:149, 1981. Briefly, the  
microplasminogen in pure or 10-fold diluted supernatants was incubated with  
urokinase for 30 minutes to activate microplasminogen to microplasmin. The  
generated microplasmin activity, as determined by its amidolytic activity measured  
25 with the chromogenic substrate S2403 (available from Chromogenix, Antwerp,  
Belgium) at different times, was compared to the activity of known amounts of  
purified plasmin or microplasmin preparations. The clone X33-MPLG1 #5, showing  
the highest microplasmin activity after urokinase activation, was selected for  
subsequent large scale production. This clone was deposited under the provisions of  
30 the Budapest Treaty with the Belgian Coordinated Collections of Microorganisms

(BCCM-MUCL-COLLECTION) on December 12, 2001, under Accession Number MUCL 43676.

(c) *Fermentation*

Fermentation of X33-MPLG1#5 at a 50 liter scale was carried out in four steps as follows. Two liter flask cell cultures were performed for 23 hours at 30°C in 400 ml YSG+ (6 g/l of yeast extract, 5 g/l of soya peptone, 20 g/l of glycerol) using an inoculum of 0.7 ml (of cell bank vial number glycerol OOC17) and 270 rpm agitation, yielding (at the end of the pre-culture step) an OD<sub>600</sub> of 15. Fermentation was then performed in a MRP80 fermentation device in 30 l basal medium (26.7 ml/l H<sub>3</sub>PO<sub>4</sub> 85%, 1.05 g/l CaSO<sub>4</sub>·2H<sub>2</sub>O, 18.2 g/l K<sub>2</sub>SO<sub>4</sub>, 14.9 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 4.13 g/l KOH, 40 g/l of 100% glycerol and 4.76 ml/l PTM1 salt solution [comprising 6 g/l CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.08 g/l NaI, 3.36 g/l MnSO<sub>4</sub>·H<sub>2</sub>O, 0.2 g/l NaMoO<sub>4</sub>·2H<sub>2</sub>O, 0.02 g/l Boric acid, 0.82 g/l CoCl<sub>2</sub>·6H<sub>2</sub>O, 20 g/l ZnCl<sub>2</sub>, 65 g/l FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g/l d-biotin and 5 ml/l HSSO<sub>4</sub>]), using 600 ml inoculum at 30°C with an air flow of 50 l/min at atmospheric pressure, dissolved oxygen (DO) >20% and 200-500 rpm agitation, pH being maintained at 5.8 with 12.5% ammonia. At 24 hours and OD 600 of 50 (end of batch step), glycerol depletion was evidenced by a rapid increase of dissolved oxygen. Glycerol feeding (632 g/l glycerol 100% and 12 ml/l PTM1) increased the OD 600 up to 258 in 24 hours. Methanol feeding was then carried out with an increasing flow of up to 250 ml/h within 6 hours, which was maintained for 66 hours using 988 ml/l methanol and 12 ml/l PTM1 to reach an OD 600 of 352 at the end of culture. Fermentation of X33-MPLG1#5 at a 350 liter scale provided proportionally similar results.

(d) *Purification*

The harvest was then purified in a three-steps process comprising cation exchange expanded bed chromatography, hydrophobic chromatography and affinity chromatography as follows:

i) Cation exchange expanded bed chromatography

Cation exchange expanded bed adsorption chromatography was conducted with Streamline SP (available from Pharmacia Biotechnology, Cat. No. 17-0993-

01/02) packed in a Streamline 200 column (Pharmacia Biotechnology Cat No. 18-1100-22) with a bed volume of 5,120 cm<sup>3</sup>, expanded and equilibrated by applying an upward flow of 1 M NaCl, 25 mM sodium acetate (CH<sub>3</sub>COONa.3 H<sub>2</sub>O) buffer, pH 6.0, for two column volumes followed by column volumes of 25 mM sodium acetate buffer, pH 6.0. The fermentation broth was on line diluted (7x) with water and passed upwards through the expanded bed at a flow rate of 1000 ml/min. Loosely bound material was washed out with the upward flow of 25 mM sodium acetate buffer pH 6.0. The column adaptor was then lowered to the surface of the sedimented bed at a height of 16.3 cm. Flow was reversed and the captured proteins eluted with 2 column volumes of 0.5 M NaCl, 25 mM sodium acetate buffer, pH 6.0. Solid ammonium sulfate was added to the eluted Streamline fraction to reach 30 % saturation (164 g ammonium sulfate per liter of eluted Streamline fraction) and the mixture was gently stirred at 4-8°C for 1 hour.

ii) Hydrophobic chromatography

Hydrophobic chromatography was conducted with Hexyl TSK 650C (available from Toso-Haas Cat. No. 19027) packed in a Vantage 180/500 column (available from Millipore, Cat. No. 87018001) with a packed volume of 2,700 cm<sup>3</sup> at 4-8°C. The eluted streamline fraction was loaded on the column at a flow rate of 38 l/hour. The column was then washed with 1.5 column volumes of 25 mM sodium acetate buffer, pH 6.0, containing 164 g/l ammonium sulfate and eluted from the column with 7 column volumes of 25 mM sodium acetate buffer, pH 6.0.

iii) Affinity chromatography

Affinity chromatography was conducted with Blue Sepharose 6 Fast Flow (available from Pharmacia Biotechnology, Cat. No. 17-0948-02/03) packed in a Vantage 130/500 column (available from Millipore, Cat. No. 87013001) with a packed volume of 3,186 cm<sup>3</sup> at 4-8°C. The eluted fraction was loaded on the column at a flow rate of 20 l/hour, and washed with one column volume of 25 mM disodium hydrogenophosphate (Na<sub>2</sub>HPO<sub>4</sub>.12 H<sub>2</sub>O) buffer, pH 7.0. The microplasminogen protein fraction was eluted from the column with 5 column volumes of 0.5 M NaCl, 25 mM di-sodium hydrogenophosphate buffer, pH 7.0, and kept frozen at -20°C. The purity of the material was above 98% as demonstrated by SDS gel electrophoresis.



(e) *Quantitative activation to and stabilization of microplasmin*

5 i) Quantitative activation

The activation of microplasminogen to microplasmin was performed at 23°C for 30 minutes at a molar ratio of 0.5% of a staphylokinase variant SY162 in 0.5 M NaCl, 25 mM di-sodium hydrogenophosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ ) buffer, pH 7.0. SY162 is a staphylokinase variant with reduced immunogenicity comprising 12  
10 amino-acid substitutions (K35A, E65Q, K74R, E80A, D82A, T90A, E99D, T101S, E108A, K109A, K130T and K135R) as compared to wild-type, as described by WO 99/40198. Solid ammonium sulfate was added to microplasmin at a final concentration of 1 M (132 g/l) and the mixture stirred at 4 - 8°C for 15 minutes.

ii) Hydrophobic chromatography

15 Hydrophobic chromatography was conducted with Phenyl Sepharose 6 Fast Flow (available from Pharmacia Biotechnology, Cat. No. 17-0965-03/05) packed in a BPG 100/500 column (available from Pharmacia Biotechnology, Cat. No. 18-1103-01) having a packed volume of 1,738  $\text{cm}^3$ , equilibrated with 4 column volumes of 25 mM  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$  buffer, pH 7.0, containing 0.1 M of the stabilizing agent,  
20 tranexamic acid (available from Bournonville Pharma, Braine-L'Alleud, Belgium) and 1 M  $(\text{NH}_4)_2\text{SO}_4$  pH 7.0, at 4-8°C. The activated product was loaded on the column at a linear flow rate of 18 l/hour and washed with 4.5 column volumes of 25 mM  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$  buffer, pH 7.0, containing 0.1 M tranexamic acid and 1 M  $(\text{NH}_4)_2\text{SO}_4$ . Microplasmin was eluted from the column at a linear flow rate of 6 l/hour  
25 with 5 column volumes of 25 mM  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$  buffer, pH 7.0, containing 0.1 M tranexamic acid and 0.7 M  $(\text{NH}_4)_2\text{SO}_4$  and equilibrated with phosphate buffered saline containing 0.1 M tranexamic acid. Staphylokinase variant SY162 was eluted from the column with 25 mM  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$  buffer, pH 7.0 containing 0.1 M tranexamic acid. This procedure removed above 99% of staphylokinase from the microplasmin  
30 peak as demonstrated with a specific ELISA assay.

iii) Concentration and diafiltration by tangential ultrafiltration

5 In this step, the eluate from step (ii) was concentrated and the buffer was exchanged for the low pH citric acid buffer. The tranexamic acid of step (ii) was removed during this step and microplasmin was stabilized by the low pH citric acid buffer.

Ultrafiltration was conducted with 2 Pellicon 2 Biomax membranes (5 kDa, 2.5  $\mu$ m, available from Millipore, Bedford, Massachusetts, Cat. No. P2B005A25) at 2-  
10 8°C. The membranes were mounted in a Pellicon 2 Process Holder connected to a Microgon Pump Cart System (available from Microgon, Laguna Hills, California). The membranes were washed with purified water and membrane integrity tested before operation. Sanitization was performed by continuous recirculation with 0.5 M NaOH for 60 minutes and with 0.1 M NaOH during 60 minutes. The sanitization step  
15 deep cleans the membrane to eliminate any potential trace of protein left on the membrane before applying the sample. The membranes were then rinsed with 5 mM citric acid, pH 3.1, until the permeate reached a pH of 3.1. The pH of the Phenyl Sepharose eluate was adjusted to 3.1 and the protein was concentrated to 4 mg/ml by ultrafiltration. Diafiltration was performed for 60 to 90 minutes against 5 volumes of  
20 5 mM citric acid, pH 3.1. Yields (expressed in grams) of three runs performed on a 50 liter fermentation apparatus are summarized in Table 2.

TABLE 2

	Run 1	Run 2	Run 3
Fermentor	220	240	ND
Streamline	50	79	130
Hexyl	36	37	ND
Blue	25	28	30
Phenyl	17	20	26
Diafiltration	-	-	22

(Key: ND : not determined)

iv) Sterile filtration (0.2  $\mu$ m)

This step was performed to ensure absence of microbial contamination.

- 5 Mannitol was added at 2-8°C to a concentration of 1.5 g/g of protein and sterile filtration performed at 23°C on a Millipak 100 filter (size 500 cm<sup>2</sup>) (available from Millipore, Cat. No. MPGL10CA3) and rinsed with about 500 ml of 5 mM citric acid, pH 3.1, with a peristaltic pump at a flow rate of 500 ml/minute. The filtrate was collected in a sterile and pyrogen free bag and stored at -20°C.

10

### EXAMPLE 3

#### Method of Preparing a Recombinant, Stabilized Miniplasmin

- About 15  $\mu$ g of the vector pPICZ $\alpha$ -KMPLG1 was digested in a 20  $\mu$ l reaction with Pme I, which linearizes the vector in the 5' AOX1 region. The linear DNA (3  $\mu$ g) was used to transform competent *Pichia pastoris* X33 cells prepared according to the manual provided in the EasySelect *Pichia* Expression kit.
- 15

The selection of high-expression strain was performed essentially as follows. Zeocin resistant transformants were selected on YPDSZ plates (as defined in example 2). Fifty isolated colonies were inoculated in 15 ml BMYZ-glycerol medium (as

defined in example 2) in 50 ml Falcon tubes and cultured for 16 hours at 30°C. The cells were pelleted and re-suspended in 1.5 ml of BMYZ-methanol medium (as defined in example 2) to induce expression from the *AOX1* promoter, and cultured for 40 hours. 3 or 4 pulses of 0.5 % methanol were regularly supplied to the cultures over this period. At the end of the induction culture, the presence of miniplasminogen in the culture supernatant was estimated as described by Lijnen et al. (cited *supra*). Briefly, the miniplasminogen in 10-fold diluted supernatants was incubated with streptokinase for 10 minutes to form an active complex. The generated miniplasmin activity, as determined with the chromogenic substrate S2403 (see example 2) at different times, was compared to the activity of known amounts of a purified plasminogen preparation. In these conditions, all tested clones produced miniplasminogen with yields varying between 3 and 15 mg/l. The two clones X33-KMPLG1 #6 and X33-KMPLG1 #25, showing the highest miniplasmin activity, were selected for subsequent large scale production. These two clones were deposited under the provisions of the Budapest Treaty with the Belgian Coordinated Collections of Microorganisms (BCCM-MUCL-COLLECTION) on December 4, 2003 and have been accorded Accession Number MUCL 45309 (clone X33-KMPLG1 #6) and Accession Number MUCL 45308 (clone X33-KMPLG1 #25).

#### EXAMPLE 4

##### Novel Method of Fixation

This experiment was carried out to establish a fixation technique that is reliable to investigate the effects of media and different agents on posterior vitreous detachment (PVD) in normal porcine eyes.

Freshly isolated porcine eyes obtained from the slaughterhouse were either immediately processed or allowed to sit at room temperature for up to 6 hours. The cornea was removed to facilitate fixation. Eyes were fixed in Peter's solution (1.25% glutaraldehyde/1% paraformaldehyde in 0.08 M cacodylate buffer pH 7.4) at 0°C for 24 to 36 hours to stop enzymatic reactions. The eyes were then washed with 0.1 M cacodylate buffer pH 7.4 and progressively dehydrated in progressively higher concentrations of ethanol up to 100%.

Both the freshly processed eyes and the eyes allowed to sit for 6 hours showed no significant change in the ultrastructure of the retina, and the vitreous remained attached to the retinal surface. Thus, this method provides a non-traumatic fixation procedure for eye tissue and minimizes the possibility of the separation of the vitreous from the retinal surface. Furthermore, this procedure allows the whole retinal surface, from the optic nerve to the retinal periphery to be studied.

#### EXAMPLE 5

##### Effect of Microplasmin on the Vitreoretinal Interface in Post-Mortem Porcine Eyes

This experiment was carried out to determine the ability of microplasmin to disinsert the posterior vitreous cortex from the inner limiting membrane of the retinal surface.

Microplasmin was used at the following doses: 0.0625, 0.125, 0.156, 0.25 and 0.390 mg, in a volume of 0.1 ml of the intraocular irrigating solution, BSS PLUS®. The pH of these microplasmin solutions ranged from 7.92 for the 0.0625 mg dose to 6.52 for the 0.390 mg dose.

The microplasmin solutions disclosed above were separately injected into the vitreous humor of eyes obtained from freshly slaughtered pigs at room temperature (24°C). The eyes were fixed as described in Example 4, after 15 minutes, 30 minutes, 60 minutes or 120 minutes following injection of microplasmin. Posterior vitreous detachment (PVD) was observed after 1 hour of treatment with 0.0625 mg of microplasmin, whereas PVD was visible from about 30 minutes after the injection for all doses including and above 0.125 mg of microplasmin. This detachment was most apparent around 120 minutes post-injection (Fig. 4, Panel A), in all sections of the retina surface, except near the vitreous base (the zone extending from the peripheral retina to the ora serrata where the adhesion to the vitreous is strong). In addition to posterior vitreous detachment (Fig. 4, Panels B-E), electron microscopy showed that the structure of the vitreous was altered to have less fibrillary structure present. The fibrillar structure was modified to a more amorphous, ground glass consistency, which indicates liquefaction of the vitreous humor (Fig. 4, Panel F).

Doses lower than 0.25 mg did not result in any ocular or retinal toxicity as determined by gross examination or histopathology, including electron microscopy. In particular, there was no sign of autolysis. Vacuolation of cells is often seen as an early sign of autolysis and at doses lower than 0.390 mg, no vacuolation was observed. We also examined other ocular structures by electron microscopy (Fig. 5, Panels A and B). No structural alterations to the retina were observed at doses lower than 0.390 mg. However, in a few eyes treated with 0.25 mg of microplasmin, retinal elevations and small numbers of inflammatory cells were sparsely distributed on the retinal surface. Gross histology of the eye treated with the highest dose of microplasmin (0.390 mg), indicated that the retinal interface had a whitish appearance. Electron microscopy of this eye revealed that there were multiple small elevations in the retinal surface, which suggests localized retinal detachment.

These experiments show that microplasmin used at a dosage of 0.06 to 0.2 mg resulted in consistent separation of the posterior hyaloid without inducing any ultrastructural changes in the retina. The posterior hyaloid separation is not only at the optic nerve but also all the way to the vitreous base. The posterior hyaloid separation leaves a clear, smooth retinal surface on which no collagen fibers can be recognized using high-electron microscopic scanning (12,000X magnification), a magnification that is high enough to exclude the possibility of undetected fibers.

20

## EXAMPLE 6

### Posterior Vitreous Detachment in Human Post-Mortem Eyes

This experiment was performed to determine whether microplasmin could efficiently induce vitreoretinal separation in human eyes.

## 25 Methods

### (a) *Dosage and Treatment of Human Post-Mortem Eyes*

Twenty-six human globes with no known eye pathology were obtained from the Munich eye bank. These globes were removed from 13 donors, whose ages ranged from 34 to 69 years, within 19 hours of their death. After harvesting the

cornea using a 14 mm diameter trephine, the 26 globes were incubated in a moist chamber at 37°C for 15 minutes. 0.2 ml of microplasmin was then injected into the vitreous cavity of thirteen eyes. Specifically, 1.25 mg of microplasmin was diluted with 4 ml, 2 ml, or 1.5 ml of the intraocular irrigating solution, BSS PLUS® to achieve concentrations of 0.3125 mg/ml, 0.625 mg/ml, and 0.9375 mg/ml respectively. A total volume of 0.2 ml of these solutions was injected into the vitreous cavity, resulting in a final dose of 62.5 µg, 125 µg, and 188 µg of microplasmin respectively within the eye. The 13 fellow eyes, which served as controls, received an injection of 0.2 ml of balanced salt solution (BSS PLUS®).

Of the 13 eyes treated with microplasmin, 9 eyes were treated by an intravitreal injection of microplasmin alone. A dose of 62.5 µg of microplasmin (pH 7.4) was injected into the vitreous cavity of two eyes; a dose of 125 µg of microplasmin (pH 7.2) was injected into the vitreous cavity of 5 eyes; and a dose of 188 µg of microplasmin (pH 7.2) was administered in 2 eyes. Of the remaining 4 eyes, two were treated with 62.5 µg of microplasmin and 0.6 ml of sulfurhexafluoride (SF<sub>6</sub>) and two were treated with 125 µg of microplasmin and 0.6 ml of sulfurhexafluoride (SF<sub>6</sub>). The additional treatment with SF<sub>6</sub> was performed because of previous reports that plasmin only induces PVD in combination with vitrectomy or gas injection. The dosing and treatment discussed above are summarized in Table 3.

TABLE 3

Number of eyes	Dose of microplasmin in µg	Treatment
2	62.5	Intravitreal injection
5	125	Intravitreal injection
2	188	Intravitreal injection
2	62.5	Intravitreal injection and gas tamponade (0.6 ml SF <sub>6</sub> )
2	125	Intravitreal injection and gas tamponade (0.6 ml SF <sub>6</sub> )

Following treatment, all eyes were incubated at 37°C for 30 minutes. After that time, the globes were placed in 4% paraformaldehyde, and 0.1 ml of fixative (4% paraformaldehyde) was also injected into the vitreous cavity to stop enzymatic action within the eye. The globes that were treated with microplasmin and SF<sub>6</sub> were fixed  
5 with the posterior pole in an upright position.

*(b) Scanning and Transmission Electron microscopy*

The globes were then hemisected along the pars plana, and the anterior segment was discarded. A corneal trephine of 12.5 mm diameter was slowly moved through the vitreous and the posterior pole was punched out. Retinal specimens for  
10 scanning and transmission electron microscopy were then obtained from the posterior pole using a corneal trephine of 4 mm diameter.

Retinal discs for scanning electron microscopy were post-fixed in 2% osmium tetroxide (Dalton's fixative), dehydrated in ethanol, dried to the critical point, sputter-coated in gold, and photographed using a ISM-35 CF electron microscope (JEOL®,  
15 Tokyo, Japan).

Specimens for transmission electron microscopy were post-fixed in Dalton's fixative, dehydrated, and embedded in EPON™. Semithin sections were stained with 2% toluidine blue. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and analyzed using a Zeiss EM 9 electron microscope (Zeiss, Jena,  
20 Germany).

Two observers independently evaluated the electron micrographs. Each observer evaluated the degree of vitreoretinal separation by deciding whether a continuous or discontinuous network of collagen fibrils covered the inner limiting membrane (ILM), or whether single or sparse collagen fibrils were present at the ILM,  
25 or whether the ILM was devoid of any collagen fibrils (bare ILM).

Results

*(a) Scanning electron microscopy*

Scanning electron microscopy (SEM) of post-mortem human eyes injected with 62.5 µg of microplasmin revealed a posterior vitreous detachment leaving a



discontinuous network of collagen fibrils covering the ILM (Fig. 6, Panel A). SEM of eyes injected with 125  $\mu$ g (Fig. 6, Panel B) and 188  $\mu$ g (Fig. 6, Panel C) of microplasmin respectively revealed a bare ILM consistent with complete vitreoretinal separation. Both these higher doses resulted in a similar ultrastructure of the

5 vitreoretinal interface.

In eyes injected with 62.5  $\mu$ g of microplasmin and SF<sub>6</sub>, remnants of cortical vitreous covering the ILM were observed (Fig. 6, Panel D). However in eyes injected with 125  $\mu$ g of microplasmin followed by gas tamponade, complete vitreoretinal separation consistent with a bare ILM was observed (Fig. 6, Panel E).

10 In contrast to the microplasmin-treated eyes discussed above, control eyes did not exhibit posterior vitreous detachment as determined by SEM (Fig. 6, Panel F). These results are summarized in Table 4.

**TABLE 4**

Dose of microplasmin in $\mu$ g	Treatment	Degree of residual cortical vitreous	
		Treated eyes	Control eyes
62.5	Intravitreal injection	++	+++
125	Intravitreal injection	-	+++
188	Intravitreal injection	-	+++
62.5	Intravitreal injection and gas tamponade	++	+++
125	Intravitreal injection and gas tamponade	-	+++

[Key: +++ continuous network of collagen fibrils; ++ discontinuous network of collagen fibrils; + sparse collagen fibrils; - no collagen fibrils, bare ILM]

15

(b) *Transmission electron microscopy*

The intraretinal morphology of all microplasmin-treated eyes was unchanged compared to control eyes. The ultrastructure of the ILM was well preserved in microplasmin-treated eyes (Figure 7, Panel A), as compared to the control eye (Figure 7, Panel B).

Conclusion

These data indicate that intravitreal injection of microplasmin can induce a cleavage between the vitreous cortex and the inner limiting membrane of the human eye without vitrectomy or any other surgical intervention. 125 µg of microplasmin cleaves the human vitreoretinal junction within 30 minutes. In terms of enzymatic action, 125 µg of microplasmin is the equivalent dose of 2 U of plasmin (Sigma-Aldrich, Munich, Germany), which caused complete vitreoretinal separation in pig cadaver eyes and in human donor eyes. These data also show that the application of a gas bubble into the vitreous of a microplasmin-treated eye did not affect the dose needed to cleave the vitreoretinal junction.

EXAMPLE 7

*In Vivo* Analysis of Microplasmin- Induced Posterior Vitreous Detachment

The purpose of these experiments was to determine the utility of microplasmin for PVD *in vivo*.

Methods

(a) *The Feline Model*

The feline retina has been extensively studied by anatomists and physiologists making this a useful model to assess the safety of pharmacologically induced PVD. Like the human retina, the cat retina is rod-dominated and has an intraretinal circulation, outside the fovea. This is in contrast to the rabbit retina, which has no intraretinal vessels. The rabbit inner retina is perfused by vasculature that lies on its vitreal surface, and this limits the value of experimental studies primarily focused on the vitreoretinal interface in the rabbit. For years, the feline model has provided high-

quality data on the cellular responses of the retina to detachment. Thus, we used a feline model to study the utility of microplasmin in effecting PVD *in vivo*.

Five adult domestic cats aged between 12 and 23 months were anesthetized with an intramuscular injection of 0.5 ml of ketamine (Ketaset, Park-Davis, Eastleigh, UK) and 0.3 ml of medetomidine (Dormitor, Pfizer, UK). The anesthetized cats received an intravitreal injection of 14.5 µg or 25 µg of microplasmin, while the fellow eyes of these cats received an injection of balanced salt solution (BSS-PLUS®) and served as controls. Of the 5 cats used in this study, 3 cats received an intravitreal injection of 25 µg of microplasmin. One of these cats was sacrificed after one day of receiving the injection; the second was sacrificed after 3 days and the third was sacrificed after 3 weeks. The two remaining cats were injected with 14.5 µg microplasmin. Of these, one was sacrificed after 3 days while the other was sacrificed after 3 weeks.

(b) *Scanning and Transmission Electron Microscopy*

The globes were removed from the sacrificed cats, fixed and processed for electron microscopy as described for human post-mortem eyes in Example 6. Electron micrographs were evaluated independently by two observers. Each observer evaluated the degree of vitreoretinal separation by deciding whether a continuous or discontinuous network of collagen fibrils covered the ILM, or whether single or sparse collagen fibrils were present at the ILM, or whether the ILM was devoid of any collagen fibrils.

(c) *Confocal microscopy*

The eye specimens were rinsed in phosphate buffered saline (PBS) and orientated in 5% agarose (Sigma, St Louis MO, USA) prepared in PBS. One hundred micrometer thick sections were cut using a vibratome (Technical Products International, Polysciences, Warrington, PA, USA) and incubated in normal donkey serum (1:20; Dianova, Hamburg, Germany) in PBS containing 0.5% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA, USA), 0.1% Triton X-100 (Roche Boehringer, Mannheim, Germany) and 0.1% sodium azide (Sigma-Aldrich, Munich, Germany) (this PBS solution containing BSA, Triton and azide is referred to as PBTA) overnight at 4°C on a rotator. After removal of blocking serum, primary

antibodies were added in six sets of pairs: anti-glial fibrillary acidic protein (GFAP; 1:500; DAKO, Hamburg, Germany) with anti-collagen IV (1:50; DAKO); anti-vimentin (1:50; DAKO) with anti-fibronectin (1:400; DAKO); anti-synaptophysin (1:50; DAKO) with anti-neurofilament (1:25; DAKO); anti-laminin (1:25; DAKO) with anti-CD 68 (1:50; DAKO); anti-red/green opsin (1:100; Santa Cruz Biotechnology, USA) with anti-rhodopsin (1:200; Santa Cruz Biotech); anti-blue opsin (1:100; Santa Cruz Biotech, Santa Cruz, CA, USA) with anti-rhodopsin (1:200; Santa Cruz Biotech). The specificity of the antibodies used in this experiment is listed in Table 5.

10

**TABLE 5**

<b>Antibody</b>	<b>Specificity</b>
Anti-glial fibrillic acidic protein (GFAP) Anti-vimentin	Intermediate filament proteins of Müller cells
Anti-neurofilament	Neurofilaments in ganglion cells and in horizontal cells
Anti-synaptophysin	Synaptic vesicles in plexiform layers
Anti-red/green opsin Anti-blue opsin	Cones
Anti-rhodopsin	Rods
Anti-fibronectin Anti-laminin	Fibronectin Laminin
Anti-collagen IV	Collagen type IV
Anti-CD68	Macrophages

After overnight incubation at 4°C on a rotator, sections were rinsed in PBTA and incubated again overnight at 4°C with the secondary antibody. Donkey anti-mouse and donkey anti-rabbit secondary antibodies were used for each combination of primary antibodies, conjugated to Cy2 or Cy3 (Dianova, Hamburg, Germany). All

secondary antibodies were used at a dilution of 1:100, and all the antibodies were diluted in PBTA. The sections were then rinsed, mounted in N-propyl gallate in glycerol and viewed on a laser scanning confocal microscope (LSM 510, Zeiss, Germany).

## 5 Results

### (a) *Scanning electron microscopy*

One day following intravitreal injection of 25  $\mu$ g microplasmin, sparse collagen fibrils covered the ILM (Figure 8, Panel A). Three days after treatment, 25  $\mu$ g microplasmin resulted in complete vitreoretinal separation (Figure 8, Panel B);  
 10 there were no remnants of collagen fibrils left on the vitreoretinal interface. The eye which received 14.5  $\mu$ g of microplasmin revealed sparse collagen fibrils covering the ILM 3 days after the injection (Figure 8, Panel C ). 21 days after treatment, with 14.5  $\mu$ g (Figure 8, Panel D ) and 25  $\mu$ g of microplasmin (Figure 8, Panel E) a bare ILM  
 15 was observed. All fellow control eyes had a dense network of collagen fibrils covering the retina (Figure 8, Panel F). These data are summarized in Table 6.

**TABLE 6**

Dose of microplasmin in $\mu$ g	Duration of treatment in days	Degree of residual cortical vitreous	
		Treated eyes	Control eyes
14.5	3	+	+++
14.5	21	-	+++
25	1	+	+++
25	3	-	+++
25	21	-	+++

[Key: +++ continuous network of collagen fibrils; ++ discontinuous network of collagen fibrils; + sparse collagen fibrils; - no collagen fibrils, bare ILM]

### (b) *Light and transmission electron microscopy*

Regarding the cytoarchitecture of the retina, no difference was observed between microplasmin-treated eyes (Figure 9, Panel A) and control eyes (Figure 9, Panel B). The ultrastructure of the inner retina and the ILM of microplasmin treated eyes (Figure 9, Panels C and E) were well preserved compared to the inner retina and  
5 the ILM of control eyes (Figure 9, Panels D and F).

(c) *Laser confocal microscopy*

In microplasmin-treated eyes and in control eyes, the end-foot portion of Müller cells was clearly labeled by anti-GFAP (Figure 10, Panels A and B) and anti-  
10 vimentin (Figure 10, Panels C and D). There was no extended staining of Müller cell processes beyond the inner nuclear layer. No significant staining was observed with anti-collagen IV and anti-fibronectin (data not shown). This may relate to species specificity of these antibodies. The ILM was stained by anti-laminin. Few  
15 macrophages were present in both treated eyes and control eyes. The ganglion cell axons and dendrites, the horizontal cells, and the inner and outer plexiform layer were clearly labeled by anti-neurofilament and anti-synaptophysin (Figure 10, Panels E and F). The photoreceptor layer was labeled by anti-neurofilament. There was no  
20 difference between microplasmin-treated eyes (Panels A, C, E) and control eyes (Fig.10, Panels B, D and F) at any time point of the study, with respect to any of the antibodies used.

Discussion

To assess the cleaving effect of microplasmin at the vitreoretinal interface *in vivo*, we administered two different doses into the vitreous cavity of five adult cats. The first dose we used was 25 µg of microplasmin, which is one-fifth of the dose that  
25 was found sufficient to induce complete PVD in human post-mortem eyes. Notably 25 µg of microplasmin is equivalent to 0.4 U of plasmin (Sigma), and clinically 0.4 U of autologous plasmin has been applied to the vitreous cavity of human eyes with  
macular holes and diabetic retinopathy. The second dose of 14.5 µg of microplasmin is equivalent to a dose of 25 µg of microplasmin in the human eye, if one adjusts for  
30 the smaller vitreous volume of the cat eye (roughly 60% of the vitreous volume of the human eye).

Three days following an intravitreal application of 25 µg of microplasmin in the cat eye, there was complete vitreoretinal separation, whereas one day after treatment some collagen fibrils were still present at the vitreoretinal interface. This indicates that the effect of microplasmin continues beyond 24 hours and stands in striking contrast to the rapid inactivation of plasmin in the blood by its natural antagonist alpha-2-antiplasmin. One reason for the longevity of microplasmin activity may be that alpha-2-antiplasmin is saturated by another substrate, or that microplasmin has a different affinity for the antiplasmin antagonist compared to plasmin. Another possible reason could be that pathways downstream of microplasmin (for example, activation of collagenases or matrix metalloproteinases) remain active after microplasmin is inactivated by alpha-2-antiplasmin.

The cytoarchitecture of the retina of microplasmin-treated eyes was unchanged compared to control eyes. In ultrastructural terms, there was no difference in the retinal anatomy between microplasmin-treated eyes and control eyes. The ILM and the retina were well preserved in all specimens. Additionally we did not observe any signs of an inflammatory reaction following microplasmin injection. Specifically, electron microscopy and laser confocal microscopy did not show any evidence of inflammatory cellular infiltration of the retina.

In the feline model, retinal detachment produces a significant proliferation of Müller cells and a massive upregulation of intermediate filament proteins in their cytoplasm, such as glial fibrillar acidic protein (GFAP) and vimentin. This Müller cell response is widely known as gliosis, and is supposed to play a key role in the complex cellular responses of the retina to detachment. In the normal retina, Müller cells appear quiescent and express very small amounts of GFAP and vimentin. However, even vitrectomy without inducing retinal detachment has been shown to cause upregulation of GFAP. Recent work by our group demonstrated marked upregulation of intermediate filament proteins following attempted peeling of the ILM in cat eyes (unpublished data). These data point to the high reactivity of Müller cells to any form of surgical trauma.

In the present study, we did not observe any change of Müller cell reactivity following induction of PVD by microplasmin. Moreover, there was no difference between treated eyes and control eyes with respect to any antibody applied. The

quiescent state of Müller cells at any time point of the study in association with the unchanged ultrastructure and immunoreactivity of the retina provides experimental evidence pointing to the safety of microplasmin in inducing PVD.

In conclusion, these studies show that microplasmin is effective in inducing PVD *in vivo*. In addition, these studies indicate that microplasmin appears safe in that no retinal alterations were observed at the ultrastructural level.

#### EXAMPLE 8

##### Evaluation of the Effects of Microplasmin on Porcine Vitreous by Use of Dynamic Light Scattering

This study was conducted to evaluate the effects of microplasmin ( $\mu$ Pli) to characterize the biophysical effects of  $\mu$ Pli on fresh, post-mortem porcine vitreous *in vitro* and *in-situ* using the non-invasive technique of dynamic light scattering (DLS). DLS provides information about the dynamics of particles and macromolecules in solutions and suspensions by measuring time fluctuations in the intensity of the scattered light.

In a DLS experiment, a constantly fluctuating speckle pattern is seen in the far field when light passes through an ensemble of small particles suspended in a fluid (*see*, Chu B., Laser light scattering: Basic principles and practice, Academic Press, New York, 1991). This speckle pattern is the result of interference in the light paths and it fluctuates as the particles in the scattering medium perform random movements on a time scale of  $\geq 1$   $\mu$ sec due to the collisions between themselves and the fluid molecules (Brownian motion). In the absence of particle-particle interactions (dilute dispersions) light scattered from small particles fluctuates rapidly while light scattered from large particles fluctuates more slowly.

The DLS apparatus built for our studies provides dynamic information such as diffusion coefficient, size, scattered intensity, and polydispersity (measure of heterogeneity). In general, an increase in particle sizes (from nanometers to a few microns) and an increase in the number or density of these particles result in an increase in scattered light intensity. Polydispersity is a measure of the number of distinct groups of species with different size(s). In a DLS measurement up to three



groups differing in size can be identified since they diffuse at different time scales (small particles move faster and larger slower). Therefore, a change in scattered light intensity and polydispersity can complement the particle size data. If after pharmacologic intervention vitreous particle sizes decrease and scattered intensity increases, then there is probably an increased number of smaller size molecular species in the solution, either due to breakdown of larger molecular species, and/or, as in the case of these experiments, an influx of 20 nm polystyrene nanospheres that were previously excluded by the inherent vitreous structure. If polydispersity decreases, then the most likely explanation is that there is increased homogeneity in the population of molecular species in the sample, again indicative of the influx of 20 nm polystyrene nanospheres that were previously excluded. The most attractive features of DLS are that it is non-invasive and quantitative, works effectively for particle sizes ranging from a few nm to few  $\mu\text{m}$ , requires small samples volume, and works reasonably well for polydisperse or multiple size (up to 2-3 component) dispersions. The data presented herein was analyzed using the cumulant and exponential size distribution routines obtained from Brookhaven Instruments, NY. These schemes have been reviewed by Stock and Ray (Stock R.S. and Ray W. H., *J. Polym. Sci.* 23:1393, 1985). As an example, Figure 11 shows a typical DLS measurement in terms of a time autocorrelation function or TCF for the whole porcine vitreous (polydisperse system) and a solution of polystyrene nanospheres of 20 nm diameter (monodisperse system).

#### Materials and Methods

##### *(a) Fabrication of DLS Apparatus for Vitreous Studies*

A new compact DLS fiber-optic probe (U.S. Patent No. 5,973,779) was fabricated for the vitreous studies described herein. It comprises of a pair of 0.25 pitch Selfoc GRIN lenses, and it has a penetration depth of ~16 mm and a scattering angle of 160°. A fiber optic probe comprising two monomode optical fibers and two GRIN lenses provides a compact and remote means of studying the dynamic characteristics of the macromolecules in the eye. Two monomode optical fibers, each housed in a stainless steel ferrule, are mounted into a separate stainless steel housing. An air gap is intentionally left between the fiber housing and the lens housing in order to produce a tightly focused spot in the scattering volume. The two optical fibers in

their housings are aligned and fixed into position off-axis with the micro lens. The two housings are placed inside a third (outer) housing made of stainless steel, and the back end of the housing is covered with a heat-shrink tubing. The two free ends of the optical fibers were terminated with FC/PC-type male connectors for easy mating with the laser and photo-detector module.

The experimental set up's main components consist of a DLS compact fiber optic probe (described above), a computer (Gateway PC 500S) containing a digital correlator card (BI-9000 Brookhaven Instruments NY), and a 635 nm wavelength 1 mW solid-state laser (OZ Optics, Canada), and an avalanche photo diode detector (Perkin Elmer, Canada). The probe is mounted on an optical assembly connected through translational stages controlled manually to access and direct the probe to a desired location in the eye.

Each temporal autocorrelation function (TCF) took 20 seconds to collect (except for the filtered cuvette studies which took 1 minute). The delay time of 5 microseconds was kept constant for all the measurements. Prior to starting the vitreous study, the instrument was thoroughly tested for its stability, reliability, and reproducibility by using aqueous dispersions of polystyrene standards (latex nanospheres of 20 nm diameter).

*(b) Dynamic light scattering of Vitreous and Viscosity Issues*

DLS is able to non-invasively provide objective quantification of the average diameter of particles suspended in a solution, in this case the vitreous. To calculate particle sizes accurately it is necessary to either know, or assume the viscosity of the solvent. As previously mentioned, it is not presently possible to accurately measure the viscosity of a non-Newtonian fluid, so assumptions must be made. Given that vitreous is approximately 99% water, it is reasonable to ascribe the viscosity of water to the vitreous. To test the validity of this assumption, DLS measurements were made on whole vitreous gel, the sub-fraction of vitreous that did not pass through a strainer (gel), the sub-fraction of vitreous that did pass through the strainer (non-gel), and the sub-fraction of vitreous that passed through a 0.22  $\mu\text{m}$  Millipore filter (liquid vitreous). These studies were done in optical cuvettes and diffusing 20 nm polystyrene nanospheres were added as a tracer with a known diameter that is highly uniform. DLS measurements in whole vitreous and all the different sub-fractions

yielded similar results indicating the vitreous microviscosity is indeed very close to that of pure water. Microviscosity in vitreous refers to the viscosity of trapped water in which the hyaluronan (HA) molecules and collagen bundles are suspended. The Brownian motion of HA molecules are much faster than the collagen bundles which are fairly large in size compared to HA molecules. In the DLS spectra, HA molecular information is embedded at faster (short) delay times and the collagen at slower (longer) delay times. The derived information is shown in the particle size distribution(s).

(c) *Reagents*

All solutions were prepared using BSS PLUS® (ALCON Labs, Ft. Worth, TX). Microplasmin was used at a concentration of 4 mg/ml stock solution. Polystyrene nanospheres (Bangs Laboratories, Fishers, IN) of 20 nm diameter and suspended in doubly distilled de-ionized water were added at a fixed ratio in all samples, assuring a uniform number of nanospheres in all samples.

(d) *Open Sky Model*

Fresh, unfixed porcine eyes (n=11) underwent dissection of the anterior segment via a pars planar incision and sharp dissection of the lens/iris diaphragm off the anterior vitreous. The dissection was carried as close to the posterior lens capsule as possible, without incising this tissue. The eyes were maintained in a holder with the posterior segment below.

The specimens were treated with controls and  $\mu$ Pli at doses of 0.08, 0.125, 0.4, 0.6 and 0.8 mg at room temperature by placing the solutions (300  $\mu$ l) that contained 60  $\mu$ L of 20 nm polystyrene nanosphere solution onto the anterior surface of the exposed vitreous body. DLS was performed at a single point along the central optical axis located 1, 2, and 4 mm below the vitreous/air interface. Every 15 minutes a DLS reading was obtained for a duration of 90 to 360 minutes.

(e) *Closed Eye Model*

A 30 G needle was used to inject 300  $\mu$ L of experimental and control solutions containing polystyrene nanospheres and  $\mu$ Pli at doses of 0.0125, 0.025, 0.05, 0.125, 0.25, 0.5, 0.6, and 0.8 mg via a stab incision at the pars plana of intact porcine eyes (n

= 39). Eyes were incubated in a holder that maintained the cornea above and the posterior segment below (mimicking the supine position) at 37 degrees Celsius for either 30 or 120 minutes. Prior to placing the eye in the temperature-controlled water bath (at all times avoiding any contact between the specimen and the water) and every 5 15 minutes thereafter, the eye was rotated about the optical axis for 10-15 seconds. After incubation, the eyes underwent excision of the anterior segment via a pars planar incision. DLS was performed at several points (mean = 18.6, s.d. =11.8) along the optical axis and along a horizontal axis (mean = 30.8 points, s.d. =18.0) at a depth of 4 mm behind the vitreous/air interface.

## 10 Results

### (a) *Molecular Morphology of Porcine Vitreous*

DLS measurements of whole (intact) vitreous obtained at multiple points along the optical axis of the eye cup (anterior segment dissected away) demonstrate very similar findings at all points from 1.25 mm to 4.75 mm below the air-vitreous 15 interface. These findings are also similar to those obtained in excised whole vitreous placed into a cuvette, in the residual vitreous retained after straining, and in the sub-fraction of vitreous that passed through the strainer. All showed nearly identical particle size distributions. The group of larger particle sizes (to the right) has an average size of about 1000 nm and represents primarily collagen. The smaller particle 20 size distribution (to the left) primarily represents hyaluronan (HA). This particle size distribution pattern was the same in eyes that received polystyrene nanosphere injection.

### (b) *Open Sky Model*

Figure 12 shows the TCF's obtained from a point 4 mm below the air/vitreous 25 interface in 5 different porcine eyes and from a solution of 20 nm polystyrene nanospheres, for comparison. It can be seen that with increasing doses of  $\mu$ Pli there is a decrease in the slope of the TCF with disappearance of the slow component (larger molecular species) ultimately approaching the TCF of pure 20 nm nanospheres, i.e. all smaller size molecular species.

The results of DLS measurements (n=5) at a depth of 1 mm following treatment with various solutions at room temperature: Placebo and 0.08 mg were essentially the same throughout. With a dose of 0.125 mg there was about a one-third reduction in the overall average particle size after 210 minutes. There was about an  
5 80% reduction in average particle size after 60 minutes and nearly complete reduction of average particle sizes (only 20 nm polystyrene nanospheres were detected) with the 0.6 mg dose.

At a measurement depth of 2 mm behind the air/vitreous interface (n=3) there was about a one-third reduction in the overall average particle size after 180 minutes  
10 (data not shown). Incubating the specimen at 37 degrees Celsius and maintaining that temperature during DLS measurements resulted in a 40% reduction in the overall average particle size after treatment with 0.5 mg  $\mu$ Pli for 60 minutes in two separate eyes at two different times. The total intensity measurements and the polydispersity measurements for all of these specimens supported and corroborated the particle size  
15 determinations.

(c) *Closed Eye Model*

After a 30-minute incubation at 37 degrees Celsius with  $\mu$ Pli doses ranging from 0.0125 to 0.8 mg there were significant changes along both the optical and horizontal axes. In the optical axis there was a seven-fold diminution in the  
20 normalized average particle size at the highest dose of 0.8 mg. The dose of 0.125mg yielded a diminution in the normalized average particle size of about one-third after 30 minutes. The lowest dose of 0.0125 mg did not appear to have any significant effects. Across the entire range of doses the diminution in particle size was inversely proportional to the dose of  $\mu$ Pli (coefficient of correlation = 0.93). The data was fitted  
25 to a linear (straight-line) fitting routine. The normalized total intensity and polydispersity plots further support the reliability of this data. The particle size distributions demonstrate a significant shift to the left in the distribution of particle sizes with increasing doses of  $\mu$ Pli. This suggests that  $\mu$ Pli is effective in significantly  
30 weakening and breaking various chemical bonds and lysing vitreous macromolecular structure. Similar changes were detected along the horizontal axis.

There were also significant changes after a 2-hour incubation at 37 degrees Celsius with  $\mu$ Pli doses ranging from 0.0125 to 0.6 mg. At the highest dose there was

an 87.5% decrease in the normalized average particle size measured along the optical axis. Normalized scattering intensity and normalized polydispersity plots corroborate this data. The particle size distributions showed a shift to the left with increasing doses, attaining significant proportions with the higher doses. DLS measurements  
5 along a horizontal axis at a depth of 4 mm showed similar results, with about an 85% diminution in the normalized average particle sizes and supportive findings on normalized scattering intensity and polydispersity plots.

Comparison of the results at 30 minutes and at 2 hours demonstrates a more extensive degree of particle size breakdown with longer incubation. Consider that at a  
10 dose of 0.6 mg the normalized average particle diameter was about 20% in the 30-minute incubation and about 10% in the 2-hour incubation. Thus, there was about a two-fold greater decrease in particle size with longer incubation.

The primary parameter that derives from the DLS measurements is the diffusion coefficient. A change in the diffusion coefficient indicates a change in the  
15 vitreous macromolecular structure in response to  $\mu$ Pli treatment. With increasing  $\mu$ Pli doses there is an increase in the diffusion coefficient of the vitreous. Across the range of  $\mu$ Pli doses, diffusion coefficients were directly proportional to  $\mu$ Pli dose. Thus, with increasing  $\mu$ Pli dose there were decreasing diffusion coefficients and, similar to particle size determinations, this correlation was statistically significant ( $r = 0.93$ ).

## 20 Discussion

In this experiment DLS was used to non-invasively assess molecular structure in vitreous by measuring particle sizes, scattering intensity, and polydispersity. The results showed that there are similar DLS profiles in various locations within whole vitreous. The most pronounced effects of microplasmin were upon whole vitreous  
25 incubated at 37 degrees C for 30 min, especially at higher doses. There was a substantial diminution in normalized average particle size and a statistically significant dose-response relation was established. This suggests that  $\mu$ Pli would be useful as an adjunct for vitreo-retinal surgery, since a 30 minute time frame is reasonable for a drug effect that does not interfere with current surgical practices. In  
30 conjunction with the data in the previous Examples suggesting that  $\mu$ Pli induces dehiscence at the vitreo-retinal interface, this drug appears to achieve the two desired components for pharmacologic vitreolysis: posterior vitreous detachment and a

breakdown in vitreous macromolecules with consequent increases in vitreous diffusion coefficients and ultimately liquefaction.

#### EXAMPLE 9

##### Microplasmin as an Adjunct to Surgical Vitrectomy

5           A patient presenting vitreoretinal disease in which surgical vitrectomy is indicated is treated with an injection of microplasmin prior to the surgical vitrectomy procedure. The patient is to receive a full ophthalmic examination to establish a baseline of ocular health. The ophthalmic examination includes indirect ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular  
10   pressure measurements, visual acuity (unaided and best corrected) symptomatology, fundus photography, fluorescein angiography, electroretinography and A-scan measurements.

          Either up to 30 minutes or up to 1 day prior to the start of vitrectomy, the eye to be treated is injected with 0.025 to 0.125 mg of microplasmin in 0.2 ml of the  
15   intraocular irrigating solution, BSS PLUS® or other irrigating solution to promote the liquefaction of the vitreous and/or induce posterior vitreous detachment.

          By promoting liquefaction of the vitreous and/or posterior vitreous detachment, the surgical vitrectomy may be made quicker and easier with less iatrogenic retinal trauma and risk of surgical complications. Allowing for more  
20   complete removal of vitreous may also lessen the risk of post-operative complications such as proliferative vitreoretinopathy.

#### EXAMPLE 10

##### Treatment of Diabetic Retinopathy with Microplasmin

25           In this Example, a diabetic patient manifesting diabetic retinopathy is treated by the intravitreal injection of microplasmin.

          The diabetic patient is to receive a full ophthalmic examination to establish a baseline of ocular health. The ophthalmic examination includes indirect

ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected) symptomatology, fundus photography, fluorescein angiography, electroretinography and A-scan measurements.

5           Following the preliminary examination, an intravitreal injection of microplasmin is given to the patient's affected eye. If both eyes are affected, they may be treated separately. The eye to be treated is injected with a dose ranging from 0.005 mg to 0.125 mg of microplasmin in 0.05 to 0.2 ml of BSS PLUS® or other irrigating solution intravitreally to promote the liquefaction of the vitreous.

10 After treatment, the patients' eyes are to be examined periodically. The extent of diabetic retinopathy presented by the patient is continuously monitored through periodic retinal examinations and fluorescein angiograms to monitor the extent of venous beading, IRMA, retinal ischemia, traction retinal detachment, vitreous hemorrhage, need for vitrectomy, or other complications of diabetic retinopathy.

15 EXAMPLE 11

### Effect of microplasmin compared to plasmin on speed of fluorescein diffusion in post-mortem pig eyes

Microplasmin is approximately one-third the molecular weight of full-length plasmin. Due to its smaller size, microplasmin is expected to diffuse more rapidly in the vitreous than plasmin (Xu, J. et al., *cited supra*). More rapid diffusion would be expected to lead to a more rapid pharmacologic effect. The present study was performed in order to confirm both that microplasmin diffuses more rapidly than plasmin, and that microplasmin is able to alter the vitreous gel.

## Methods

25 Freshly isolated porcine eyes obtained from the slaughterhouse were used. In the first experiment, one eye was injected with microplasmin (0.125mg) and the fellow eye with vehicle control (BSS-PLUS®). After maintaining both eyes at room temperature for 2 hours, both eyes were then injected with fluorescein and incubated an additional 30 minutes. Photographs were taken at time 0, 10, 20, and 30 minutes.



In the second experiment, eyes were injected with microplasmin 0.125 mg (N=2) or plasmin 1U (supplied by Sigma, N=2), and incubated at 37 degrees Celsius for 2 hours. All 4 eyes were then injected with fluorescein and incubated for an additional 30 minutes, with photographs taken at time 0, 10, 20, and 30 minutes.

## 5 Results

In the first experiment, in the control eye virtually no fluorescein diffusion within the vitreous was observed (data not shown). However, in the microplasmin-treated eye clear fluorescein diffusion was observed (data not shown).

In the second experiment, microplasmin-treated eyes had fluorescein diffusion of 14% and 16%, respectively, over 20 minutes (Figure 13), while the plasmin-treated eye had fluorescein diffusion of less than 10% (Figure 14).

## Discussion

Microplasmin demonstrated a clear facilitation of fluorescein diffusion compared to vehicle control. Furthermore, as predicted based on molecular weight of microplasmin, this fluorescein diffusion was of a greater extent than that observed with full-length plasmin administration. These findings support the theoretical prediction that microplasmin diffuses more rapidly than plasmin. These findings may have clinical benefit, in allowing for more rapid pharmacologic effect.

What is claimed is:

1. A method of treating or preventing a disorder, or a complication of a disorder, of an eye of a subject comprising contacting a vitreous and/or an aqueous humor with an effective amount of a composition comprising a truncated plasmin protein
- 5 comprising a catalytic domain of plasmin (TPCD).
2. The method of claim 1, wherein the TPCD has a molecular weight less than about 40,000 daltons.
3. The method of claim 1, wherein the TPCD has a molecular weight between about 20,000 and 30,000 daltons.
- 10 4. The method of claim 1, wherein the TPCD has a molecular weight of about 26,500 daltons in reduced form or about 29,000 daltons in non-reduced form.
5. The method of claim 1, wherein the TPCD has a molecular weight less than about 20,000 daltons.
6. The method of claim 1, wherein said TPCD is miniplasmin.
- 15 7. The method of claim 1, wherein said TPCD is stabilized miniplasmin.
8. The method of claim 1, wherein said TPCD is recombinant miniplasmin.
9. The method of claim 1, wherein said TPCD is stabilized, recombinant miniplasmin.
10. The method of claim 1, wherein said TPCD is microplasmin.
- 20 11. The method of claim 1, wherein said TPCD is stabilized microplasmin.
12. The method of claim 1, wherein said TPCD is recombinant microplasmin.
13. The method of claim 1, wherein said TPCD is stabilized, recombinant microplasmin.
14. The method of claim 1, wherein said TPCD is a variant of microplasmin.
- 25 15. The method of claim 1, wherein the disorder of the eye is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic

- 5 vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia,
- 10 16. The method of claim 1, wherein the method reduces the viscosity of the vitreous.
17. The method of claim 1, wherein the method induces posterior vitreous detachment.
18. The method of claim 1, wherein the method reduces hemorrhagic blood from the vitreous and/or aqueous humor.
19. The method of claim 1, wherein the method reduces intraocular foreign substances from the vitreous and/or aqueous humor.
- 15 20. The method of claim 1, wherein the method increases the diffusion of an agent or a composition administered to the vitreous and/or aqueous humor.
21. The method of claim 1, wherein the method decreases extraretinal neovascularization.
22. The method of claim 1, wherein the composition is a liquid solution, and  
20 wherein the step of contacting the vitreous and/or the aqueous humor with the composition comprises injecting the liquid solution into the vitreous and/or the aqueous humor.
23. The method of claim 1, wherein the subject is a human.
24. The method of claim 1, wherein the method is performed in the absence of  
25 vitrectomy.
25. The method of claim 1, wherein the method is performed as an adjunct to vitrectomy.

26. The method of claim 1, wherein an effective amount of TPCD is in the range of 0.005 mg to 0.2 mg.

27. A method of treating or preventing a disorder, or a complication of a disorder of the eye of a subject, wherein the disorder of the eye is selected from the group  
5 consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia,  
10 endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof, comprising contacting a vitreous and/or aqueous humor with an effective amount of a composition comprising recombinant microplasmin or variants thereof, wherein the method results in vitreous liquefaction and/or posterior vitreous detachment.

15 28. A method of treating or preventing a disorder, or a complication of a disorder of the eye of a subject, wherein the disorder of the eye is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular  
20 pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof, comprising contacting a vitreous and/or aqueous humor with an effective amount of a composition comprising stabilized, recombinant microplasmin  
25 or variants thereof, wherein the method results in vitreous liquefaction and/or posterior vitreous detachment.

29. A method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject, wherein the disorder of the eye is selected from the group  
consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous  
30 hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein

occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma and retinitis pigmentosa, and any combination thereof, comprising contacting a vitreous and/or aqueous humor with an effective amount of a composition comprising recombinant miniplasmin or variants thereof, wherein the method results in vitreous liquefaction and/or posterior vitreous detachment.

30. A method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject, wherein the disorder of the eye is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof, comprising contacting a vitreous and/or aqueous humor with an effective amount of a composition comprising stabilized, recombinant miniplasmin or variants thereof, wherein the method results in vitreous liquefaction and/or posterior vitreous detachment.

31. A method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject comprising contacting a vitreous and/or aqueous humor with a composition comprising at least two TPCDs, wherein the eye disorder is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof.

32. The method of claim 31, wherein the at least two TPCDs are selected from the group consisting of miniplasmin, recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin,

recombinant microplasmin, stabilized microplasmin, stabilized, recombinant microplasmin, variants of microplasmin and any combinations thereof.

33. A method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject comprising contacting a vitreous and/or an aqueous humor with  
5 a first composition comprising at least one TPCD, and a second composition comprising at least one TPCD, wherein the eye disorder is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular  
10 pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof.

34. The method of claim 33 wherein the first composition comprising at least one  
15 TPCD and the second compositions comprising at least one TPCD are selected from the group consisting of miniplasmin, recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin, recombinant microplasmin, stabilized microplasmin, stabilized, recombinant microplasmin, variants of microplasmin and any combinations thereof.

20 35. The method of claim 33, wherein the first composition comprising at least one TPCD and the second compositions comprising at least one TPCD are administered to the subject at substantially the same time or at different times.

36. A method of treating or preventing a disorder, or a complication of a disorder, of the eye of a subject comprising contacting a vitreous and/or aqueous humor with a  
25 composition comprising at least one TPCD and at least one second agent, wherein the eye disorder is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular  
30 edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof.

37. The method of claim 36, wherein the TPCD is selected from the group consisting of miniplasmin, recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin, recombinant microplasmin, stabilized microplasmin, stabilized, recombinant microplasmin, variants of microplasmin and any combinations thereof, and wherein the second agent is selected from the group consisting of hyaluronidase, dispase, chondroitinase, collagenase, RGD containing peptides, anti-integrin antibody, urea, hydroxyurea, thiourea, P2Y receptor agonists, angiogenic inhibitors, VEGF inhibitors, PlGF inhibitors and any combinations thereof.
38. A method of performing a vitrectomy in a subject comprising a step of contacting a vitreous and/or an aqueous humor with an effective amount of a composition comprising at least one TPCD.
39. The method of claim 38, wherein the contacting step is performed prior to the vitrectomy.
40. The method of claim 38, wherein the contacting step is performed at the same time as the vitrectomy.
41. The method of claim 38, wherein the TPCD is selected from the group consisting of miniplasmin, recombinant miniplasmin, stabilized miniplasmin, stabilized, recombinant miniplasmin, variants of miniplasmin, microplasmin, recombinant microplasmin, stabilized microplasmin, stabilized, recombinant microplasmin, variants of microplasmin and any combinations thereof.
42. The method of claim 38, wherein the subject is a human.
43. The method of claim 38, wherein the vitrectomy is performed to treat or prevent a disorder, or a complication of a disorder of an eye, wherein the disorder of the eye is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal

hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof.

44. The method of claim 38, wherein the vitrectomy is carried out for a purpose selected from the group consisting of reducing the viscosity of the vitreous, liquefying the vitreous, inducing posterior vitreous detachment, clearing or reducing hemorrhagic blood from the vitreous, clearing or reducing intraocular foreign substances from the vitreous, clearing or reducing materials toxic to the retina, increasing diffusion of an agent or a composition administered to the vitreous and/or aqueous humor, decreasing extraretinal neovascularization and any combinations thereof.
45. The method of claim 38, wherein the composition is a liquid solution, and wherein the step of contacting a vitreous or aqueous humor with the composition comprises injecting the liquid solution into the vitreous or aqueous humor.
46. The method of claim 38, wherein an effective amount of TPCD is in the range of 0.005 mg to 0.2 mg.
47. A method of performing a vitrectomy in a subject comprising a step of contacting a vitreous and/or aqueous humor of an eye of the subject with an effective amount of a composition comprising a stabilized, recombinant microplasmin, before removal of the vitreous, and wherein the vitrectomy is performed to treat or prevent a disorder, or a complication of a disorder of the eye of the subject, wherein the disorder of the eye is selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof.
48. A method of performing a vitrectomy in a subject comprising a step of contacting a vitreous and/or aqueous humor of an eye of the subject with an effective amount of a composition comprising a stabilized, recombinant miniplasmin, before removal of the vitreous, and wherein the vitrectomy is performed to treat or prevent a disorder, or a complication of a disorder of the eye, wherein the disorder of the eye is



selected from the group consisting of retinal detachment, retinal tear, vitreous hemorrhage, diabetic vitreous hemorrhage, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, macular holes, vitreomacular traction, macular pucker, macular exudates, cystoid macular edema, fibrin deposition, retinal vein occlusion, retinal artery occlusion, subretinal hemorrhage, amblyopia, endophthalmitis, retinopathy of prematurity, glaucoma, retinitis pigmentosa, and any combination thereof.

49. A method of liquefying the vitreous of a subject comprising injecting a solution comprising an effective amount of a stabilized, recombinant microplasmin into the vitreous and/or aqueous humor of the subject.

50. A method of liquefying the vitreous of a subject comprising injecting a solution comprising an effective amount of a stabilized, recombinant miniplasmin into the vitreous and/or aqueous humor of the subject.

51. A method of inducing posterior vitreous detachment in an eye of a subject, comprising injecting an effective amount of a solution comprising a stabilized, recombinant microplasmin into the vitreous and/or aqueous humor of the subject.

52. A method of inducing posterior vitreous detachment in an eye of a subject, comprising injecting a solution comprising an effective amount of a stabilized, recombinant miniplasmin into the vitreous and/or aqueous humor of the subject.

53. A method of decreasing extraretinal neovascularization in an eye of a subject, comprising injecting a solution comprising an effective amount of a stabilized, recombinant microplasmin into the vitreous and/or aqueous humor of the subject.

54. A method of decreasing extraretinal neovascularization in an eye of a subject, comprising injecting a solution comprising an effective amount of a stabilized, recombinant miniplasmin into the vitreous and/or aqueous humor of the subject.

55. A composition comprising at least two TPCDs:

56. A composition comprising at least one TPCD and at least one second agent.

FIGURE 1

gag Glu 1	cct Pro	ctg Leu	gat Asp	gac Asp 5	tat Tyr	gtg Val	aat Asn	acc Thr	cag Gln 10	ggg Gly	gct Ala	tca Ser	ctg Leu	ttc Phe 15	agt Ser	48
gtc Val	act Thr	aag Lys	aag Lys 20	cag Gln	ctg Leu	gga Gly	gca Ala	gga Gly 25	agt Ser	ata Ile	gaa Glu	gaa Glu	tgt Cys 30	gca Ala	gca Ala	96
aaa Lys	tgt Cys	gag Glu 35	gag Glu	gac Asp	gaa Glu	gaa Glu	ttc Phe 40	acc Thr	tgc Cys	agg Arg	gca Ala	ttc Phe 45	caa Gln	tat Tyr	cac His	144
agt Ser	aaa Lys 50	gag Glu	caa Gln	caa Gln	tgt Cys	gtg Val 55	ata Ile	atg Met	gct Ala	gaa Glu	aac Asn 60	agg Arg	aag Lys	tcc Ser	tcc Ser	192
ata Ile 65	atc Ile	att Ile	agg Arg	atg Met	aga Arg 70	gat Asp	gta Val	gtt Val	tta Leu	ttt Phe 75	gaa Glu	aag Lys	aaa Lys	gtg Val	tat Tyr 80	240
ctc Leu	tca Ser	gag Glu	tgc Cys	aag Lys 85	act Thr	ggg Gly	aat Asn	gga Gly	aag Lys 90	aac Asn	tac Tyr	aga Arg	ggg Gly	acg Thr 95	atg Met	288
tcc Ser	aaa Lys	aca Thr	aaa Lys 100	aat Asn	ggc Gly	atc Ile	acc Thr	tgt Cys 105	caa Gln	aaa Lys	tgg Trp	agt Ser	tcc Ser 110	act Thr	tct Ser	336
ccc Pro	cac His	aga Arg 115	cct Pro	aga Arg	ttc Phe	tca Ser	cct Pro 120	gct Ala	aca Thr	cac His	ccc Pro	tca Ser 125	gag Glu	gga Gly	ctg Leu	384
gag Glu	gag Glu 130	aac Asn	tac Tyr	tgc Cys	agg Arg	aat Asn 135	cca Pro	gac Asp	aac Asn	gat Asp	ccg Pro 140	cag Gln	ggg Gly	ccc Pro	tgg Trp	432
tgc Cys 145	tat Tyr	act Thr	act Thr	gat Asp	cca Pro 150	gaa Glu	aag Lys	aga Arg	tat Tyr	gac Asp 155	tac Tyr	tgc Cys	gac Asp	att Ile	ctt Leu 160	480
gag Glu	tgt Cys	gaa Glu	gag Glu	gaa Glu 165	tgt Cys	atg Met	cat His	tgc Cys	agt Ser 170	gga Gly	gaa Glu	aac Asn	tat Tyr	gac Asp 175	ggc Gly	528
aaa Lys	att Ile	tcc Ser	aag Lys 180	acc Thr	atg Met	tct Ser	gga Gly	ctg Leu 185	gaa Glu	tgc Cys	cag Gln	gcc Ala	tgg Trp 190	gac Asp	tct Ser	576
cag Gln	agc Ser	cca Pro 195	cac His	gct Ala	cat His	gga Gly	tac Tyr 200	att Ile	cct Pro	tcc Ser	aaa Lys	ttt Phe 205	cca Pro	aac Asn	aag Lys	624

aac Asn	ctg Leu 210	aag Lys	aag Lys	aat Asn	tac Tyr	tgt Cys 215	cgt Arg	aac Asn	ccc Pro	gat Asp	agg Arg 220	gag Glu	ctg Leu	cgg Arg	cct Pro	672
tgg Trp 225	tgt Cys	ttc Phe	acc Thr	acc Thr	gac Asp 230	ccc Pro	aac Asn	aag Lys	cgc Arg	tgg Trp 235	gaa Glu	ctt Leu	tgc Cys	gac Asp	atc Ile 240	720
ccc Pro	cgc Arg	tgc Cys	aca Thr	aca Thr 245	cct Pro	cca Pro	cca Pro	tct Ser	tct Ser 250	ggg Gly	ccc Pro	acc Thr	tac Tyr	cag Gln 255	tgt Cys	768
ctg Leu	aag Lys	gga Gly	aca Thr 260	ggg Gly	gaa Glu	aac Asn	tat Tyr	cgc Arg 265	ggg Gly	aat Asn	gtg Val	gct Ala	gtt Val 270	acc Thr	gtt Val	816
tcc Ser	ggg Gly	cac His 275	acc Thr	tgt Cys	cag Gln	cac His	tgg Trp 280	agt Ser	gca Ala	cag Gln	acc Thr	cct Pro 285	cac His	aca Thr	cat His	864
aac Asn	agg Arg 290	aca Thr	cca Pro	gaa Glu	aac Asn	ttc Phe 295	ccc Pro	tgc Cys	aaa Lys	aat Asn	ttg Leu 300	gat Asp	gaa Glu	aac Asn	tac Tyr	912
tgc Cys 305	cgc Arg	aat Asn	cct Pro	gac Asp	gga Gly 310	aaa Lys	agg Arg	gcc Ala	cca Pro	tgg Trp 315	tgc Cys	cat His	aca Thr	acc Thr	aac Asn 320	960
agc Ser	caa Gln	gtg Val	cgg Arg	tgg Trp 325	gag Glu	tac Tyr	tgt Cys	aag Lys	ata Ile 330	ccg Pro	tcc Ser	tgt Cys	gac Asp	tcc Ser 335	tcc Ser	1008
cca Pro	gta Val	tcc Ser	acg Thr 340	gaa Glu	caa Gln	ttg Leu	gct Ala	ccc Pro 345	aca Thr	gca Ala	cca Pro	cct Pro	gag Glu 350	cta Leu	acc Thr	1056
cct Pro	gtg Val	gtc Val 355	cag Gln	gac Asp	tgc Cys	tac Tyr	cat His 360	ggg Gly	gat Asp	gga Gly	cag Gln	agc Ser 365	tac Tyr	cga Arg	ggc Gly	1104
aca Thr	tcc Ser 370	tcc Ser	acc Thr	acc Thr	acc Thr	aca Thr 375	gga Gly	aag Lys	aag Lys	tgt Cys	cag Gln 380	tct Ser	tgg Trp	tca Ser	tct Ser	1152
atg Met 385	aca Thr	cca Pro	cac His	cgg Arg	cac His 390	cag Gln	aag Lys	acc Thr	cca Pro	gaa Glu 395	aac Asn	tac Tyr	cca Pro	aat Asn	gct Ala 400	1200
ggc Gly	ctg Leu	aca Thr	atg Met	aac Asn 405	tac Tyr	tgc Cys	agg Arg	aat Asn	cca Pro 410	gat Asp	gcc Ala	gat Asp	aaa Lys	ggc Gly 415	ccc Pro	1248
tgg Trp	tgt Cys	ttt Phe	acc Thr 420	aca Thr	gac Asp	ccc Pro	agc Ser	gtc Val 425	agg Arg	tgg Trp	gag Glu	tac Tyr	tgc Cys 430	aac Asn	ctg Leu	1296

aaa Lys	aaa Lys	tgc Cys 435	tca Ser	gga Gly	aca Thr	gaa Glu	gcg Ala 440	agt Ser	gtt Val	gta Val	gca Ala	cct Pro 445	ccg Pro	cct Pro	gtt Val	1344
gtc Val	ctg Leu 450	ctt Leu	cca Pro	gat Asp	gta Val	gag Glu 455	act Thr	cct Pro	tcc Ser	gaa Glu	gaa Glu 460	gac Asp	tgt Cys	atg Met	ttt Phe	1392
ggg Gly 465	aat Asn	ggg Gly	aaa Lys	gga Gly	tac Tyr 470	cga Arg	ggc Gly	aag Lys	agg Arg	gcg Ala 475	acc Thr	act Thr	gtt Val	act Thr	ggg Gly 480	1440
acg Thr	cca Pro	tgc Cys	cag Gln	gac Asp 485	tgg Trp	gct Ala	gcc Ala	cag Gln	gag Glu 490	ccc Pro	cat His	aga Arg	cac His	agc Ser 495	att Ile	1488
ttc Phe	act Thr	cca Pro	gag Glu 500	aca Thr	aat Asn	cca Pro	cgg Arg	gcg Ala 505	ggg Gly	ctg Leu	gaa Glu	aaa Lys	aat Asn 510	tac Tyr	tgc Cys	1536
cgt Arg	aac Asn	cct Pro 515	gat Asp	ggg Gly	gat Asp	gta Val	ggg Gly 520	ggg Gly	ccc Pro	tgg Trp	tgc Cys	tac Tyr 525	acg Thr	aca Thr	aat Asn	1584
cca Pro	aga Arg 530	aaa Lys	ctt Leu	tac Tyr	gac Asp	tac Tyr 535	tgt Cys	gat Asp	gtc Val	cct Pro	cag Gln 540	tgt Cys	gcg Ala	gcc Ala	cct Pro	1632
tca Ser 545	ttt Phe	gat Asp	tgt Cys	ggg Gly	aag Lys 550	cct Pro	caa Gln	gtg Val	gag Glu	ccg Pro 555	aag Lys	aaa Lys	tgt Cys	cct Pro	gga Gly 560	1680
agg Arg	gtt Val	gtg Val	ggg Gly	ggg Gly 565	tgt Cys	gtg Val	gcc Ala	cac His	cca Pro 570	cat His	tcc Ser	tgg Trp	ccc Pro	tgg Trp 575	caa Gln	1728
gtc Val	agt Ser	ctt Leu	aga Arg 580	aca Thr	agg Arg	ttt Phe	gga Gly	atg Met 585	cac His	ttc Phe	tgt Cys	gga Gly	ggc Gly 590	acc Thr	ttg Leu	1776
ata Ile	tcc Ser	cca Pro 595	gag Glu	tgg Trp	gtg Val	ttg Leu	act Thr 600	gct Ala	gcc Ala	cac His	tgc Cys	ttg Leu 605	gag Glu	aag Lys	tcc Ser	1824
cca Pro	agg Arg 610	cct Pro	tca Ser	tcc Ser	tac Tyr	aag Lys 615	gtc Val	atc Ile	ctg Leu	ggg Gly	gca Ala 620	cac His	caa Gln	gaa Glu	gtg Val	1872
aat Asn 625	ctc Leu	gaa Glu	ccg Pro	cat His	gtt Val 630	cag Gln	gaa Glu	ata Ile	gaa Glu	gtg Val 635	tct Ser	agg Arg	ctg Leu	ttc Phe	ttg Leu 640	1920
gag Glu	ccc Pro	aca Thr	cga Arg	aaa Lys 645	gat Asp	att Ile	gcc Ala	ttg Leu	cta Leu 650	aag Lys	cta Leu	agc Ser	agt Ser	cct Pro 655	gcc Ala	1968

[illegible]

FIGURE 2

																gcc Ala	cct Pro	1632
tca Ser 545	ttt Phe	gat Asp	tgt Cys	ggg Gly	aag Lys 550	cct Pro	caa Gln	gtg Val	gag Glu	ccg Pro 555	aag Lys	aaa Lys	tgt Cys	cct Pro	gga Gly 560			1680
agg Arg	gtt Val	gtg Val	ggg Gly	ggg Gly 565	tgt Cys	gtg Val	gcc Ala	cac His	cca Pro 570	cat His	tcc Ser	tgg Trp	ccc Pro	tgg Trp 575	caa Gln			1728
gtc Val	agt Ser	ctt Leu	aga Arg 580	aca Thr	agg Arg	ttt Phe	gga Gly	atg Met 585	cac His	ttc Phe	tgt Cys	gga Gly	ggc Gly 590	acc Thr	ttg Leu			1776
ata Ile	tcc Ser	cca Pro 595	gag Glu	tgg Trp	gtg Val	ttg Leu	act Thr 600	gct Ala	gcc Ala	cac His	tgc Cys	ttg Leu 605	gag Glu	aag Lys	tcc Ser			1824
cca Pro	agg Arg 610	cct Pro	tca Ser	tcc Ser	tac Tyr	aag Lys 615	gtc Val	atc Ile	ctg Leu	ggg Gly	gca Ala 620	cac His	caa Gln	gaa Glu	gtg Val			1872
aat Asn 625	ctc Leu	gaa Glu	ccg Pro	cat His	gtt Val 630	cag Gln	gaa Glu	ata Ile	gaa Glu	gtg Val 635	tct Ser	agg Arg	ctg Leu	ttc Phe	ttg Leu 640			1920
gag Glu	ccc Pro	aca Thr	cga Arg	aaa Lys 645	gat Asp	att Ile	gcc Ala	ttg Leu	cta Leu 650	aag Lys	cta Leu	agc Ser	agt Ser	cct Pro 655	gcc Ala			1968
gtc Val	atc Ile	act Thr	gac Asp 660	aaa Lys	gta Val	atc Ile	cca Pro	gct Ala 665	tgt Cys	ctg Leu	cca Pro	tcc Ser	cca Pro 670	aat Asn	tat Tyr			2016
gtg Val	gtc Val	gct Ala 675	gac Asp	cgg Arg	acc Thr	gaa Glu	tgt Cys 680	ttc Phe	atc Ile	act Thr	ggc Gly	tgg Trp 685	gga Gly	gaa Glu	acc Thr			2064
caa Gln	ggg Gly 690	act Thr	ttt Phe	gga Gly	gct Ala	ggc Gly 695	ctt Leu	ctc Leu	aag Lys	gaa Glu	gcc Ala 700	cag Gln	ctc Leu	cct Pro	gtg Val			2112
att Ile 705	gag Glu	aat Asn	aaa Lys	gtg Val	tgc Cys 710	aat Asn	cgc Arg	tat Tyr	gag Glu	ttt Phe 715	ctg Leu	aat Asn	gga Gly	aga Arg	gtc Val 720			2160
caa Gln	tcc Ser	acc Thr	gaa Glu	ctc Leu 725	tgt Cys	gct Ala	ggg Gly	cat His	ttg Leu 730	gcc Ala	gga Gly	ggc Gly	act Thr	gac Asp 735	agt Ser			2208



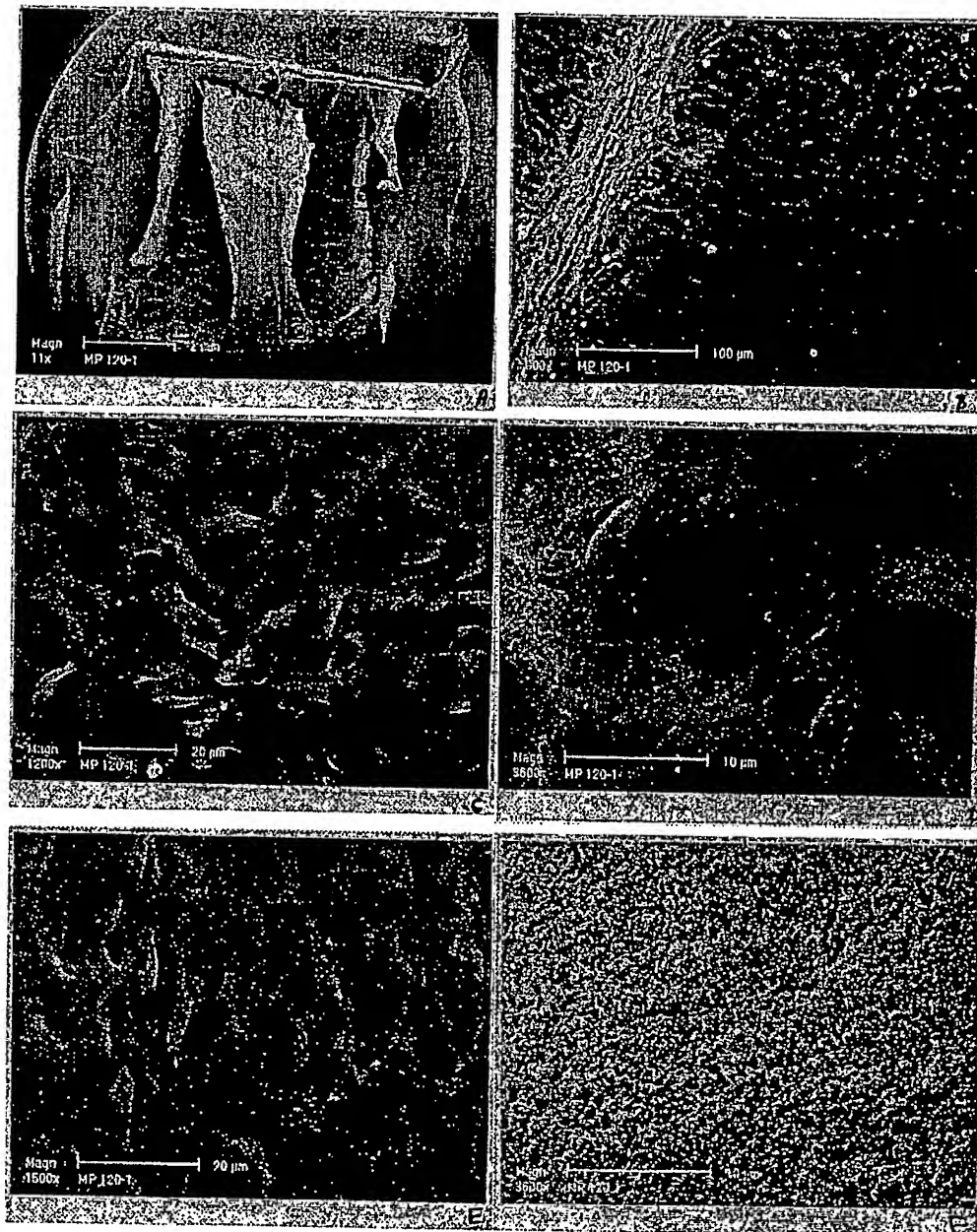
FIGURE 3

											gca Ala	cct Pro 445	ccg Pro	cct Pro	gtt Val	1344
gtc Val	ctg Leu 450	ctt Leu	cca Pro	gat Asp	gta Val	gag Glu 455	act Thr	cct Pro	tcc Ser	gaa Glu	gaa Glu 460	gac Asp	tgt Cys	atg Met	ttt Phe	1392
ggg Gly 465	aat Asn	ggg Gly	aaa Lys	gga Gly	tac Tyr 470	cga Arg	ggc Gly	aag Lys	agg Arg	gcg Ala 475	acc Thr	act Thr	gtt Val	act Thr	ggg Gly 480	1440
acg Thr	cca Pro	tgc Cys	cag Gln	gac Asp 485	tgg Trp	gct Ala	gcc Ala	cag Gln	gag Glu 490	ccc Pro	cat His	aga Arg	cac His	agc Ser 495	att Ile	1488
ttc Phe	act Thr	cca Pro	gag Glu 500	aca Thr	aat Asn	cca Pro	cgg Arg	gcg Ala 505	ggg Gly	ctg Leu	gaa Glu	aaa Lys	aat Asn 510	tac Tyr	tgc Cys	1536
cgt Arg	aac Asn	cct Pro 515	gat Asp	ggg Gly	gat Asp	gta Val	ggg Gly 520	ggg Gly	ccc Pro	tgg Trp	tgc Cys	tac Tyr 525	acg Thr	aca Thr	aat Asn	1584
cca Pro	aga Arg 530	aaa Lys	ctt Leu	tac Tyr	gac Asp	tac Tyr 535	tgt Cys	gat Asp	gtc Val	cct Pro	cag Gln 540	tgt Cys	gcg Ala	gcc Ala	cct Pro	1632
tca Ser 545	ttt Phe	gat Asp	tgt Cys	ggg Gly	aag Lys 550	cct Pro	caa Gln	gtg Val	gag Glu	ccg Pro 555	aag Lys	aaa Lys	tgt Cys	cct Pro	gga Gly 560	1680
agg Arg	gtt Val	gtg Val	ggg Gly	ggg Gly 565	tgt Cys	gtg Val	gcc Ala	cac His	cca Pro 570	cat His	tcc Ser	tgg Trp	ccc Pro	tgg Trp 575	caa Gln	1728
gtc Val	agt Ser	ctt Leu	aga Arg 580	aca Thr	agg Arg	ttt Phe	gga Gly	atg Met 585	cac His	ttc Phe	tgt Cys	gga Gly 590	ggc Gly 590	acc Thr	ttg Leu	1776
ata Ile	tcc Ser	cca Pro 595	gag Glu	tgg Trp	gtg Val	ttg Leu	act Thr 600	gct Ala	gcc Ala	cac His	tgc Cys	ttg Leu 605	gag Glu	aag Lys	tcc Ser	1824
cca Pro	agg Arg 610	cct Pro	tca Ser	tcc Ser	tac Tyr	aag Lys 615	gtc Val	atc Ile	ctg Leu	ggg Gly	gca Ala 620	cac His	caa Gln	gaa Glu	gtg Val	1872
aat Asn 625	ctc Leu	gaa Glu	ccg Pro	cat His	gtt Val 630	cag Gln	gaa Glu	ata Ile	gaa Glu	gtg Val 635	tct Ser	agg Arg	ctg Leu	ttc Phe	ttg Leu 640	1920



[illegible]

FIGURE 4



**FIGURE 5**

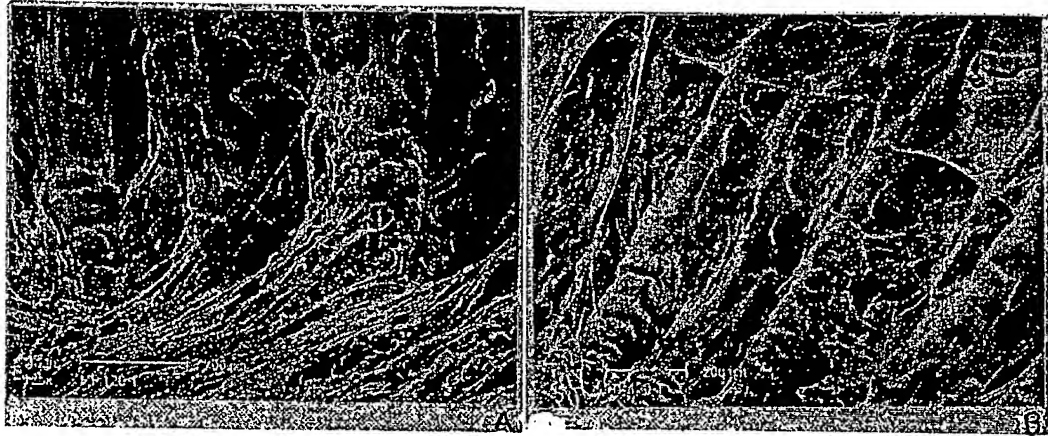


FIGURE 6

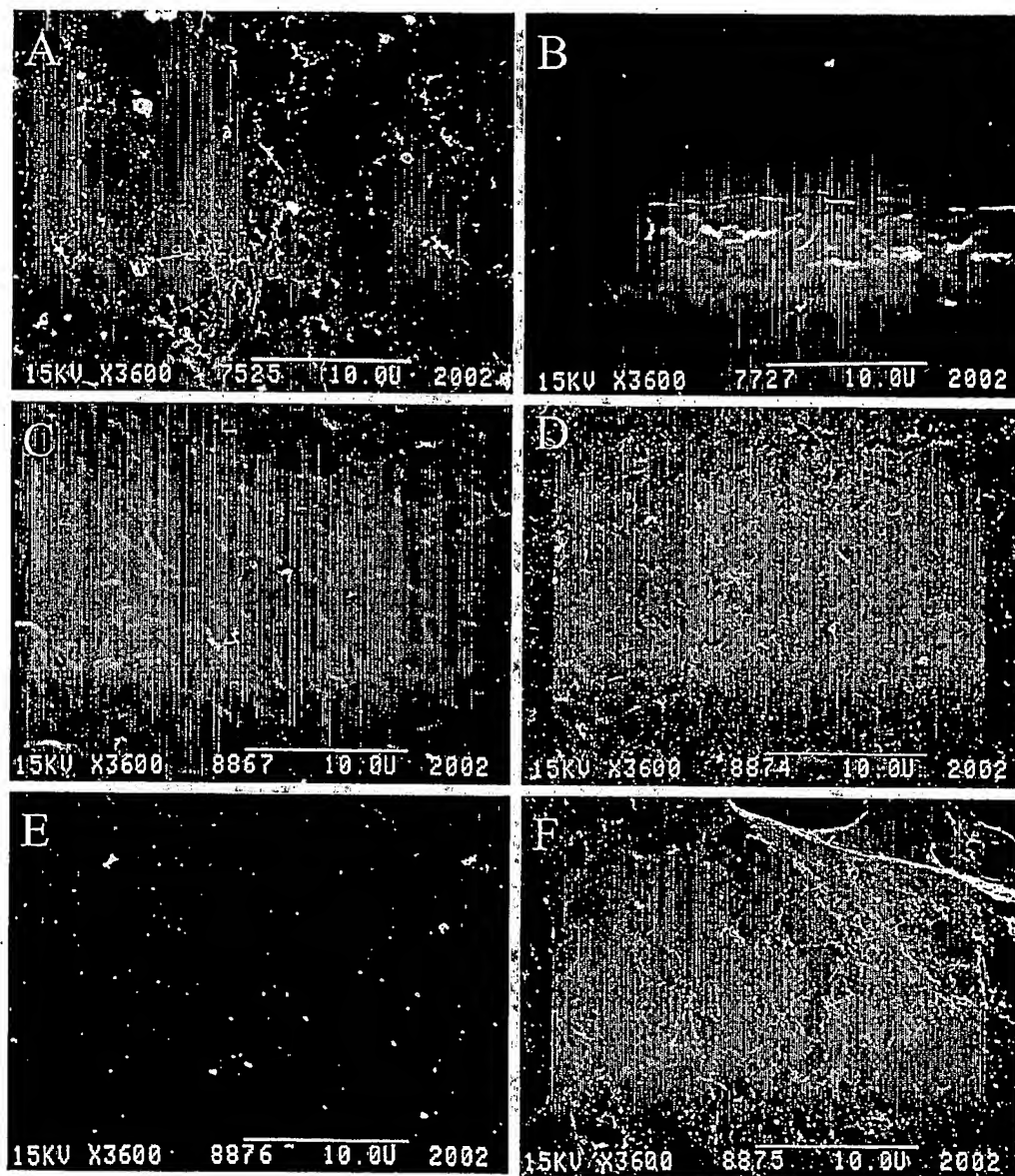


FIGURE 7



FIGURE 8

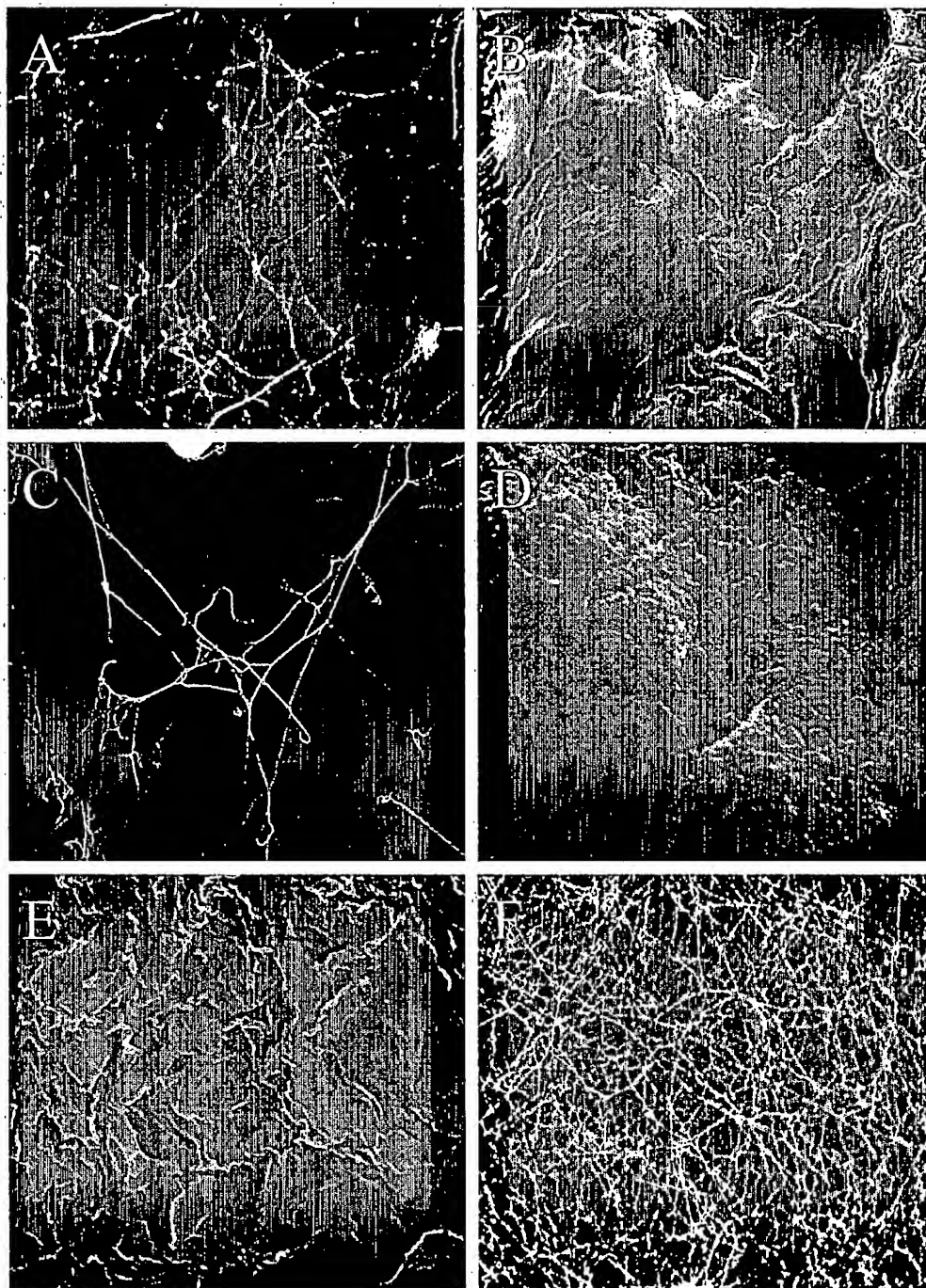


FIGURE 9

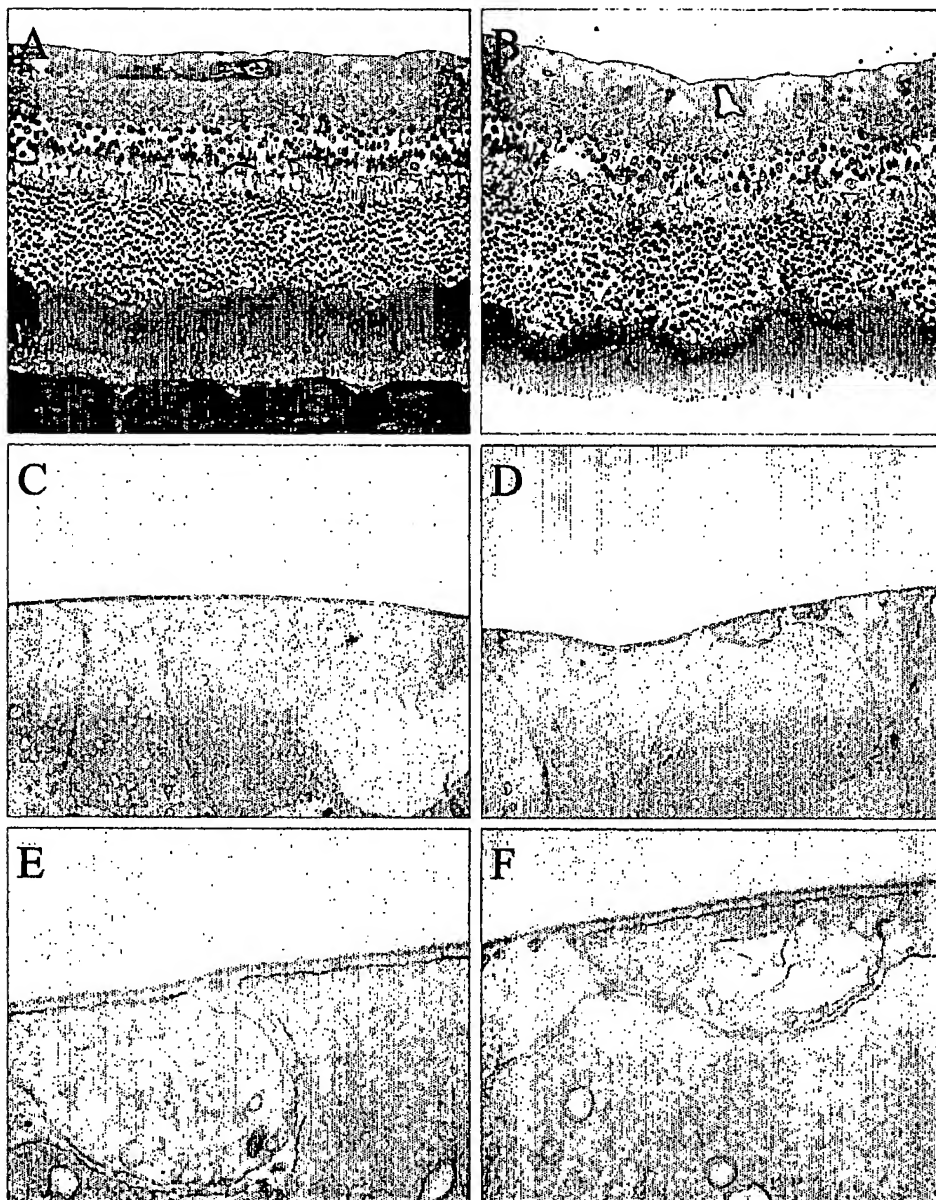


FIGURE 10

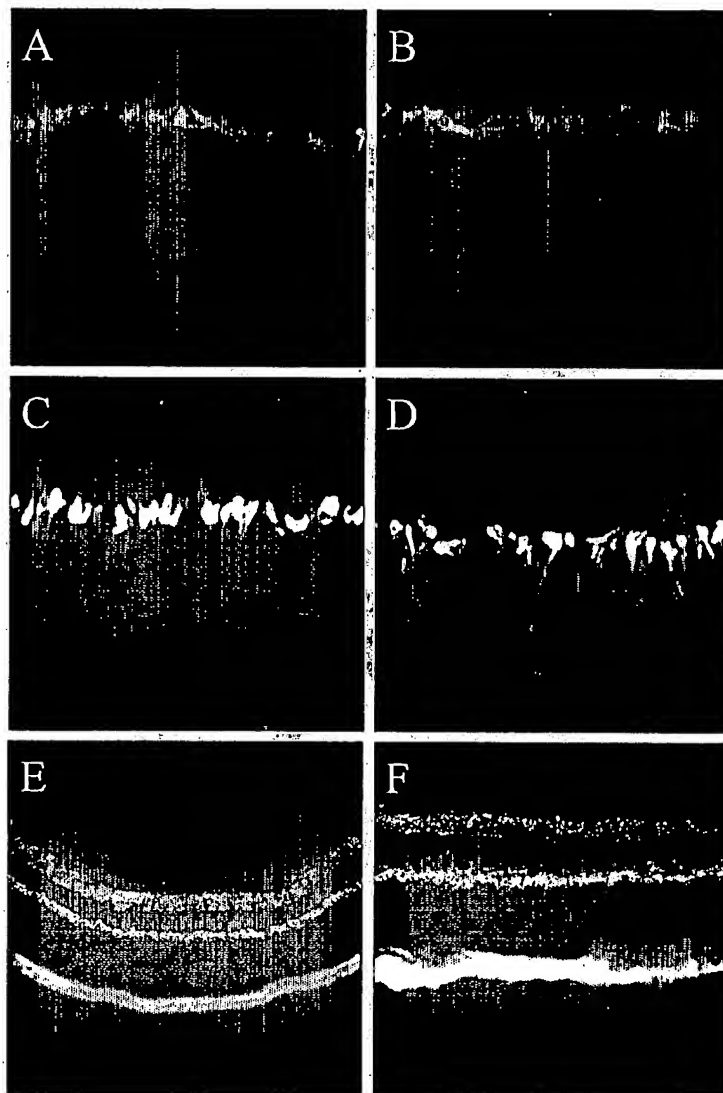




FIGURE 11

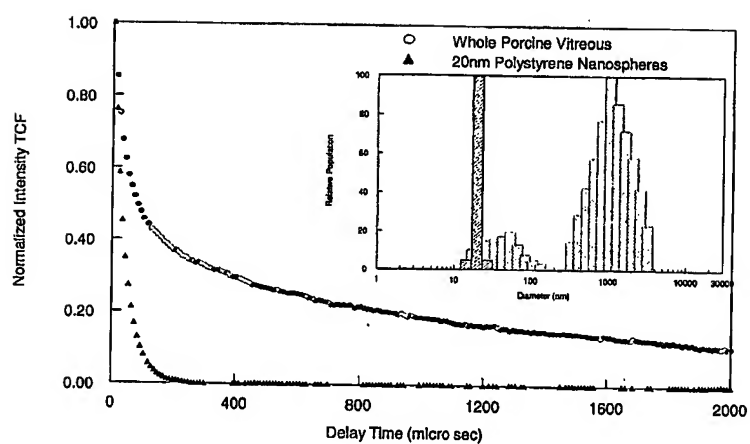


FIGURE 12

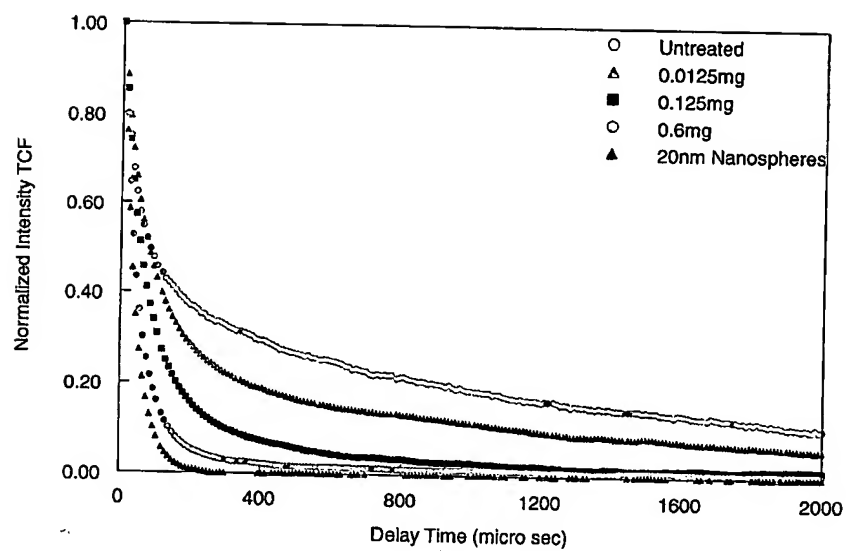


FIGURE 13

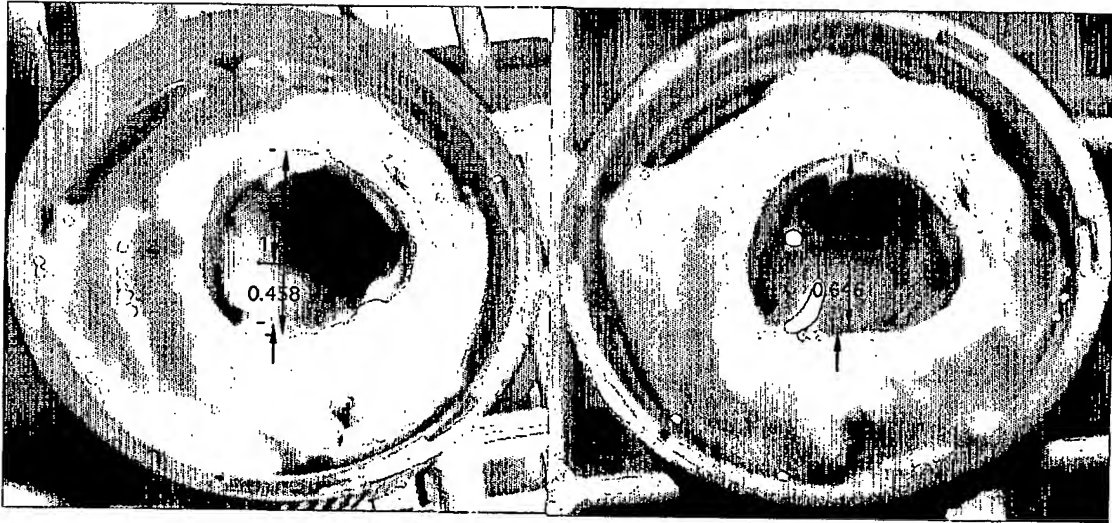


FIGURE 14

